STUDIES OF PARAMETERS INFLUENCING CELL AND PRODUCT YIELDS IN BACTERIAL CULTIVATIONS

by

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NO MAN IS AN ISLAND, ENTIRE OF ITSELF,
EVERY MAN IS A PIECE OF THE CONTINENT,
A PART OF THE MAIN .....

John Donne (1573-1631)

LIST OF PAPERS

This thesis is based on the following papers which are referred to in the text by their Roman numerals.

I. Häggström, M.H. and Dostâlek, M. Growth of Methylomonas methanolica: Factors influencing growth yield. (submitted)

II. Häggström, M.H. Growth of Streptococcus lactis on single or mixed carbohydrates in relation to the regulation of maltose metabolism. (submitted)

III. Häggström, M.H. Heterofermentative product formation in Streptococcus lactis and the regulation of glycolysis in maltose metabolism. (submitted)

IV. Häggström, M.H. and Andersson, E. Production of L- and D-lactate by Pediococcus pentosaceus in batch and steady-state continuous culture. (submitted)

V. Häggström, M.H. and Dostâlek, M. Regulation of a mixed culture of Streptococcus lactis and Saccharomycopsis fibuliger. (submitted)

VI. Leung, J.C., Häggström, M.H., Cooney, C.L. and Sinskey, A.J. Steady-state and transient growth of Streptococcus mutans in continuous culture (submitted)

and part of the review

VII. Cooney, C.L., Koplove, H.M. and Häggström, M.H. (1981) Transient phenomena in continuous culture. In: Continuous cultures of cells, Calcott, P.H. (ed). CRC Press, Florida, USA (in press).

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INTRODUCTION

In fermentation processes for the production of biomass or metabolic products the cell and product yields, respectively, are important parameters.

In the production of biomass by aerobic organisms the conversion efficiency of substrate carbon to cellular carbon is of great importance for the economy of the process. In aerobic microorganisms carbon dioxide is normally the only end product of energy metabolism, whereas in anaerobic fermentative organisms one or more products are formed. Product yields in fermentative organisms give information on the relative amounts as well as the total amount of products formed from the carbohydrate source consumed. Cell yields of an obligate methylotroph Methylomonas methanolica and product yields by three lactic bacteria (Streptococcus lactis, S.mutans, Pediococcus pentosaceus) belonging to the same family are discussed in this thesis.

Methylotrophic microorganisms can utilize compounds containing one carbon atom (C_1-compounds) (2) as carbon energy source. These organisms are used in the production of single-cell protein (SCP) from methanol and methane. The cell yield is of great importance for the economy of the process, since the cost of raw material accounts for around 50 % of the total cost of production (12, 52).

S.lactis belongs to the N-streptococci, which are used in starter cultures for the production of various fermented dairy products. P.pentosaceus and other pediococci are used in starter cultures for the production of dry sausages and occur in fermented vegetables. S.mutans is found in the oral cavity and is involved in the causation of caries. The streptococci and pediococci are regarded as homofermentative lactic acid bacteria and lactic acid is normally the main product of the energy metabolism. But under certain environmental conditions some of these organisms may be heterofermentative.

The objectives of the work presented in this thesis were to

identify parameters affecting cell or product yields in the above organisms and to elucidate the mechanisms involved in the regulation and control at the metabolic level.

The thesis also includes a survey of the transient behavior of cultures following environmental change (VII) and a discussion in relation to the transient studies carried out in the investigations I, II, III and VI. Transient-state studies can be used for identifying parameters of possible importance in the performance of bacterial cultivations when fermentation processes are scaled-up. Large scale fermenters have other mixing characteristics than bench scale fermenters and gradients are apt to develop. The transient technique is also useful in the elucidation of mechanisms involved in the control and regulation of micro-organisms at the metabolic level.

The metabolic studies of S.lactis were applied to a mixed culture of S.lactis and Saccharomycopsis fibuliger and starch was the carbohydrate supplied. Mixed cultures consist of two or more organisms and several interactions between the component strains are possible (e.g. commensalism, competition, mutualism) (29). These are involved in determining the stability of the mixed cultures, especially in continuous operation. Complex carbohydrates, such as e.g. starch or cellulose, cannot be metabolized by all microorganisms. It would thus be possible to extend the substrate availability for a number of organisms by using mixed cultures. In this context mixed cultures may be a complement or an alternative to genetic modifications of organisms.

TRANSIENTS IN RESPONSE TO ENVIRONMENTAL CHANGE VII

INTRODUCTION

Attention is directed to transient behavior in continuous culture and its effects on cell mass and product formation. The main part is a review of the literature which is followed by a discussion in relation to the transient studies reported in the papers in this thesis.

The chemostat has been used extensively for studying transient state behavior of cultures at both the population and metabolic level. A study of transient response of microorganisms is a useful source of information on mechanisms regulating growth and metabolism. Transient studies are also important in understanding the dynamic behavior of continuous cultures which is necessary for scale-up and operation of fermentation processes. Transient and oscillatory phenomena in continuous culture can be initiated by interactions between component species in mixed populations of organisms in an otherwise constant environment, or by changes in the environment. The focus here is primarily on transient response to environmental changes.

LITERATURE REVIEW

Nutritional changes

Nutritional changes which result in transient behavior of a culture can involve:

(i) step changes (shifts) in dilution rate (e.g. medium addition rate at constant volume)
(ii) pulse addition of one or more nutrients
(iii) change in feed medium concentration or composition

According to Monod, (53, 54) changes in substrate concentration(s) will effect the specific growth rate (μ) according to

the relationship

$$\mu = \mu_{max} \cdot S/(K_s + S)$$

where μ_{max} is the maximum specific growth rate (h^{-1}), K_s the half rate constant ($mg \cdot l^{-1}$) and S the limiting substrate concentration ($mg \cdot l^{-1}$). Thus, according to this model, growth is regulated by the limiting substrate and the quantitative effect of nutritional changes will be determined by the ratio of S/K_s. The cells are thus in this model assumed to have the capacity for maximum growth rate even under conditions of slow growth.

Dilution rate changes

Mateles et al. (48) and Ryu (65) examined the transient response of Escherichia coli after shifts in dilution rate in a nitrogen-limited chemostat. They found that the magnitude and the direction of the shifts were important factors in determining the response of the culture. A large increase in dilution rate (> 0.2 h^{-1}) resulted in a transient period of several hours during which the limiting substrate concentration increased before the new growth rate was reached. Small changes in dilution rate (< 0.2 h^{-1}) resulted in an immediate adjustment by the cells to the new growth rate. These results suggested that the organisms had a reserve biosynthetic capacity which would allow for a small (< 0.2 h^{-1}) immediate increase in growth rate. It is important to note from their work that the Monod model does not describe growth as a function of nutrient concentration under transient conditions. This conclusion was also confirmed by Cooney and Wang (13) in studies with Enterobacter aerogenes grown under nitrogen and nitrogen-phosphate limitation. The Monod model predicts a faster response to environmental changes than actually occurs; suggesting that the mechanism of growth limitation is not the same under both steady state and transient conditions.

For instance, Koga and Humphrey (37) have shown that variation in cell yield on the limiting nutrient can lead to damped oscilla-

tions before a new steady-state is reached after a disturbance. Studying Saccharomyces cerevisiae in a nitrogen-limited chemostat, Gilley and Bungay (23) observed damped oscillations after a shift-up in dilution rate from 0.125 to 0.270 h^{-1}; small changes in the yield coefficient could be observed. They reported that the time required for the oscillations to damp out was a function of the magnitude of the dilution rate shift, dilution rate range, and limiting nutrient concentration.

Mor and Fiechter (55) using a carbon-limited chemostat of S.cerevisiae followed several parameters during the transient period following a shift-up in dilution rate. They observed damped oscillations in cell and substrate concentration, oxygen uptake rate and carbon dioxide release at low dilution rates (< 0.07 h^{-1}). At higher dilution rates (0.10 - 0.17 h^{-1}), however, oscillations were seen in the gas exchange rates but not in other parameters. The lower the dilution rate, the lower was the rate of damping. The difference in results from those obtained by Gilley and Bungay (23) may be explained by the fact that the magnitude of the shift used was higher at the low dilution rates than at the higher dilution rates.

The behavior of cells during transients following shifts in dilution rate has been further characterized. Regan and Roper (63) showed for S.cerevisiae that a step increase in dilution rate induced budding and partial cell synchrony. Meers (50) studied the response of a mixed culture of Bacillus subtilis and Torula utilis; a shift-up in dilution rate in a magnesium-limited chemostat resulted in replacement of T.utilis by B.subtilis but the yeast outgrew the bacterium at lower growth rates. Lee et al. (39) observed similar changes in mixed cultures of Lactobacillus plantarum and Propionibacterium shermanii after shifts in dilution rate. A mixed culture of Acetobacter suboxydans and S.carlsbergensis showed oscillations in both substrate and cell concentrations after a step increase in dilution rate (9). In this study, the limiting carbon source, mannitol, was oxidized by the bacterium to fructose which was consumed by the yeast. No fluctuations were observed when the same step increase was performed in

a pure culture of S.suboxydans but, in the mixed culture, the oscillations in fructose and yeast concentration were the most pronounced. These results indicate that the instability of the mixed-culture may be caused by a feedback mechanism from the yeast to the bacterium. Cunningham and Maas (17) reported damped oscillations in cell number following a rapid change in dilution rate in a nitrogen-limited chemostat of the green flagellate Clamydomonas reinhardii. During the transient period, the specific growth rate and the average amount of limiting nutrient per cell oscillate with about the same period but with a phase lag. They hypothesized a partial uncoupling of the system controlling cell division from changes in environmental limiting nutrient concentration. Cell division appeared to be controlled by the amount of intracellular nitrogen. Storage of nitrogen may occur at high growth rates and there is a time lag between fluctuations in intracellular nitrogen content and in growth rate.

Pulse additions of limiting nutrients

A second method to induce transients in continuous culture is through the pulse addition of the limiting nutrient to temporarily remove the nutrient limitation. Assuming that all other nutrients are in excess, this type of stress will permit the cell to respond at its maximum possible rate, dictated not initially by a nutrient concentration, but by some intracellular constraint.

Welles and Blanch (80) conducted pulse feeding experiments in an anaerobic glucose-limited culture of S.cerevisiae. They showed that by continuous pulse feeding at a frequency of 0.25 (cycles/min) they could increase the ethanol yield from glucose by 50 % above the value obtained in continuous steady-state carbon-limited culture. These results may be explained by the budding phase which dominates at high growth rates leads to a higher energy requirement; this means a greater flux of ethanol production and will result in an increased product yield. The results may also be explained according to a hypothesis by Brookes and Meers (6) that more energy may be required for the continual synthesis and

degradation of enzymes which are necessary for the survival of the organisms in a changing environment. An increased ethanol yield was also observed during conditions of periodic feeding to an anaerobic glucose-limited culture of S.carlsbergensis. The increased yields were obtained when the pulse frequency used was of the same magnitude as the natural frequency of the autonomous oscillator phosphofructokinase in the cell (7). Similar studies have been carried out by Vairo et al. (78, 5) to simulate transient responses observed in domestic sewage plants. Periodic variations in feeding mash concentration or dilution rate (one cycle per 24 h) resulted in cyclic variations in cell yield and specific growth rate. However, the average yield coefficient for cell mass formation was essentially equal to the yield coefficient obtained during steady-state growth. The average specific growth rate during the transients also equaled the dilution rate.

Leung and co-workers (42) have studied the transient growth behavior of a glucose-limited chemostat of Streptococcus mutans after a pulse of glucose. The transient response is characterized by a rapid increase in production of lactic acid. The maximum specific productivity observed during the transient was dependent on the growth rate prior to the glucose pulse with faster growing cultures exhibiting a greater increase in specific productivity than slow growing cultures. After the pulse, the yield coefficient for lactic acid (g/lactic acid/g glucose) increased from a steady-state value averaging 0.4 to around 0.9.

In the preceeding, it was shown that pulse feeding of nutrients may offer an exciting possibility for increasing product yields, e.g. on ethanol and lactic acid production from glucose. However, there are also disadvantages associated with pulse or discontinuous feeding of nutrients. The increase in product yields observed in the anaerobic fermentations described above also results in a decrease in cell yield. In studies with a methanol-limited chemostat of Pseudomonas methylotropha, Brookes and Meers (6) observed lower cell yields when discontinuous medium feeding was used when compared with continuous medium addition. When the time between nutrient additions was 100 s the cell yield

fell to about 0.35 g cell mass/g methanol from an average yield of 0.44 when using continuous nutrient feed; at the same time, the yield of carbon dioxide increased. The pool amino acids, glutamine and glutamate, also varied during the feeding cycle. The authors discuss two possible explanations for the observed decrease in cell yield. First, that the organism, which had been shown to give higher yields when grown under carbon-limited conditions than when carbon is present in excess methanol. Second, that the continuous readjustment of levels of enzyme and key intermediates in a response to the changing environment requires increased energy and decreases the energy available for cell synthesis. Luscombe (44) however, did not observe a decrease in cell yield in a continuous culture of Arthrobacter globiformis fed medium in a discontinuous manner. The results, however, may not be contradictory since Luscombe (44) did pulse experiments with long intervals (120 s) between the medium additions and at a very low dilution rate ($0.01\ h^{-1}$) where the yield coefficient is already low. The possibility that an effect on cell yield would be observed using shorter intervals between the additions at higher dilution rates therefore cannot be ruled out.

Pulse additions have also been used by Cooney and Wang (13) in studying the transient response of Enterobacter aerogenes to additions of ammonia to a culture with dual nutrient (nitrogen and phosphate) limitations. When ammonia was the only growth-limiting nutrient, the organisms were not capable of responding to a pulse addition of ammonia with their theoretical maximum rate as defined by the Monod model. The response pattern showed that the cells exhibited an initial rapid response and may have a reserve biosynthetic capacity which permits immediate adjustment to small changes. Similar responses have been observed in other transient studies involving step changes in dilution rate (48, 67). An increasing restriction in phosphate availability decreased the ability of the cell to respond to the ammonia pulse. With a dual nitrogen and phosphate limitation, the initial response observed after the pulse addition of ammonia were absent and as the limiting phosphate concentration was decreased, the ability of the cells to respond to ammonia pulses was decreased. The

biosynthetic capacity which had been seen under conditions of phosphate excess were felt to be decreased by the phosphate limitation.

Koch and co-workers (35, 36) have demonstrated that E.coli has a reserve biosynthetic capacity in nitrogen-, phosphate- and carbon-limited chemostats. After a nutritional shift-up, the organism showed an immediate initial response in protein synthesizing capacity after which they slowly adjusted to the final required level. A sulphate-limited culture did not show this reserve biosynthetic capacity. They postulated a connection between the ability to respond to a change and the RNA synthesizing capacity of the cell.

Step changes in the limiting nutrient concentration

Transient behavior, similar to that observed after dilution rate shifts occurs when the feed medium composition is changed. A step increase in the limiting substrate concentration in the feed medium causes a transient increase in specific growth rate after which it returns to the original specific growth rate but at a new and higher cell density.

Regan et al. (63) investigated the behavior of Saccharomyces cerevisiae in a glucose-limited chemostat after changes in glucose concentration in the feed medium. The cell concentration reached its new steady state through a series of steps approximately one generation apart. This observation supported the authors' hypothesis that the step induced a certain degree of synchrony by encouraging the onset of budding. Budding has been shown to be energy intensive and accompanied by rapid utilization of carbohydrate. After the step in glucose supply rate, a large increase in energy production and a small increase in specific glucose consumption rate was observed, this also supported their hypothesis.

Young and Bungay (82) also studied transients following a step

change in feed glucose concentration in a chemostat culture of S.cerevisiae. They demonstrated a close relationship between cellular RNA content and the specific growth rate.

Pickett et al. (61) studied the transient response to a continuous square-wave perturbation in the limiting substrate concentration to a chemostat of E.coli. A high frequency pulsing decreased the yield but increased the protein content of the cell. The authors discussed Koch's (101) suggestion that E.coli was containing both active and inactive ribosomes where the latter would be activated when nutrient supply is increased. After a shift-down in nutrient supply a part of the functional ribosomes would be deactivated. It was proposed that if activation was faster than deactivation, a frequency of nutrient cycling shorter than the ribosomal deactivation time would be expected to increase the number of active ribosomes and thus the synthetic capacity of the cell, resulting in a higher protein content.

Temperature change

There are several reports on transient behavior in continuous culture following shifts in temperature. Topiwala and Sinclair (75) studied the transient response after temperature shifts in continuous culture with Klebsiella aerogenes; they observed a lag before the cell growth was affected and a new steady-state reached. These authors developed a model to describe their experimental results. The model worked but when the temperature was replaced by an effective temperature, the effective temperature is related to the culture temperature by a first order lag with a time constant.

Ryu and Mateles (66) observed a similar lag between the time of a temperature shift and the time when the transient maximum or minimum growth rate is reached. After temperature shift-up and shift-down experiments in a nitrogen-limited chemostat of E.coli the growth rate passed through maximum and minimum values respectively before reaching a new steady-state. Larger shifts resulted in

greater differences between the transient maximum or minimum and the new steady-state growth rate. Further, the magnitude of the transient growth rate was less than would be expected if they followed an Arrhenius function. Both Topiwala (75) and Ryu and Mateles (66) offered explanations in terms of physiological readjustment during the lags in the transient response. Ryu and Mateles (66) proposed that only a fraction of the ribosomal RNA is involved in protein synthesis under steady-state conditions and reserves can be activated to counteract the effect of sudden environmental changes. Further, they suggested that the ribosome content of the organism must be adjusted to compensate for the decrease or increase in ribosomal activity resulting from the shift in temperature.

Harder and Veldkamp (26) studying a phychrophilic Pseudomonas sp found that the RNA content in this organism was a function of growth temperature with a minimum at the optimum growth temperature (14^{o} C). They considered the increased RNA content at suboptimal temperature to be a way for the organism to compensate for slower reaction rates. However, the RNA content also increased at temperatures above temperature optimum. They studied the properties of RNA at low and high temperatures by following the transient response of a chemostat to a simultaneous shift in temperature and dilution rate. Cells growing at 10^{o} and 18^{o} C and dilution rate 0.05 h^{-1} and cells growing at 14^{o} C and dilution rate 0.10 h^{-1} had the same RNA content. In a shift from 10^{o} C and dilution rate 0.05 h^{-1} to 14^{o} C and dilution rate 0.10 h^{-1}, the cells were able to continue growth without adjusting the cellular RNA concentration. A shift from 18^{o} C and dilution rate 0.05 h^{-1} to 14^{o} C and dilution rate 0.10 h^{-1} resulted in a drop in cell concentration during the first few hours during which the cellular RNA concentration increased. This eventually resulted in an increase in growth rates and cell concentration. RNA synthesized at 18^{o} C though present in sufficient quantity is not appropriate to serve the need for higher rate of protein synthesis required at the higher growth rate. It was also shown that RNA synthesized at 18^{o} C supported a lower rate of protein synthesis compared with RNA synthesized at 10^{o} C.

Matsché and Andrew (47) performed transient experiments using a thermophilic Bacillus sp. and compared theirs and others experimental results with theoretical curves obtained using a model based on the Arrhenius relationship and with an introduction of a first order lag for the response of the growth rate. In an experiment where the temperature was shifted from 60 to 52° C, the cell concentration gradually increased to a new steady-state value and the simulation fitted the experimental curve quite well. When the temperature was shifted from 52 to 60° C, the model failed to predict the response. A sharp decrease in growth rate was observed accompanied by a transient accumulation of glucose. This behavior may be explained by rapid cell death and lysis of the cells. Their simulation studies using data from work by Ryu and Mateles (66) fitted fairly well to the experimental curve. Temperature shift experiments giving similar results as described above have also been carried out by Sterkin et al. (68).

Inhibitor addition

There are a few reports where transient response in continuous culture has been used to study the effect of inhibitors or toxic materials on growth. Zines and Rogers (83) studied the transient behavior of a product-limited culture in a turbidostat with Klebsiella aerogenes. The specific growth rate rapidly decreased after the pulse addition of the product inhibitor, ethanol. After the pulse, a decrease in the rate of total acid production, oxygen uptake rate and rate of carbon dioxide release were also observed. The culture initially exhibited a similar response to step changes in ethanol concentration in the feed medium. However, the growth rate passed through a minimum and showed damped oscillations before reaching a new steady-state value. The final growth rate attained was higher than the minimum transient and indicates adaptation of the culture to ethanol. The new steady-state values for oxygen and carbon dioxide productions were higher and the rate of acid production was lower after the step addition of ethanol. Ethanol seems to stimulate the respiratory activity and inhibit the fermentative ability of the culture. The authors also proposed

a mathematical model for the transient behavior of a product-limited system.

In Candida utilis the cytochrome content of the cells transiently increased following a pulse addition of the uncoupler 2,4-dinitrophenol or ethanol indicating that uncoupling also is involved in the latter case. The mechanism proposed was a derepression of cytochrome genes based on changes in the ADP/ATP ratio of the cell (8). This mechanism agree with the findings of Katz et al. using Torulopsis utilis where ethanol decreased the oxidative phosphorylation efficiency (33).

Zines and Rogers (84) have also applied frequency response analysis to study the effect of ethanol on the dynamic characterization of K.aerogenes. A nitrogen-limited chemostat was exposed to sinusoidal variations in dilution rate using a medium with or without ethanol. These experiments showed that ethanol significantly increased the time constant for the rate of total acid production and caused in general a slower metabolic response.

The unsteady-state continuous culture was used as an experimental tool by Borzani and Vairo (4) for studying microbial inhibition. Using S.cerevisiae, they examined the transient response after pulse, as well as step additions of the inhibitor potassium sorbate. The specific growth rate of the yeast was drastically reduced when the inhibitor loading (g inhibitor/g cell) reached a critical value regardless of the kind of disturbance used.

Lee et al. (40) examined the Lactobacillus plantarum growing aerobically in a glucose-limited chemostat and observed damped oscillations in cell and hydrogen peroxide concentration at low dilution rates ($D \leq 0.14\ h^{-1}$) after the start of continuous flow. The period and the amplitude of the oscillations increased with decreased values of dilution rate. The production and oscillation in the concentration of hydrogen peroxide which is known to have an inhibitory effect on lactobacilli suggests that it may play a major role leading to the oscillations. The oscillations do not occur at high dilution rates. After a shift in dilution rate from

0.28 to 0.08 h^{-1}, oscillations in cell and hydrogen peroxide concentration developed.

Carbon catabolite repression

Transient behavior in a carbon-limited chemostat with Escherichia coli growing on a mixture of carbon sources has been studied by Standing et al. (67). E.coli was exposed to a shift-up in dilution rate and substrate changes. When switched from glucose to xylose, the cell population decreased and xylose transiently accumulated in the medium before growth on xylose proceeded. Repeated shifts of the same kind showed that xylose metabolizing activity is lost during the period of growth on glucose; this behavior can be explained by catabolite repression. A preferential utilization of glucose was observed after a shift-up in dilution rate when the organism was grown on glucose and xylose or glucose and galactose. Xylose or galactose transiently accumulates in the medium after such a shift.

Summary

Studies on transient response in continuous culture have shown that microorganisms have only a limited "reserve biosynthetic capacity" which will allow for an immediate increase in growth rate. This capacity is dependent on the nature of growth limitation (38, 56) and is restricted further under conditions of dual nutrient limitation (82). It has been demonstrated that sudden nutritional or temperature changes may induce an oscillatory response in cell, substrate and product concentrations with the magnitude of the shift being an important factor influencing the response pattern.

Several studies have shown that there is a close relationship between the RNA content of the cell and the specific growth rate (26, 14, 30). The RNA content and its ability to support protein synthesis has also been reported to vary with temperature (26).

These results implicate ribosomal-RNA as a determinant of the biosynthetic capacity of the cell.

During transients induced by nutritional shift changes in cell and product yield coefficients have been reported. The cell yield decreases and the yields of products of energy metabolism increase. One explanation of this response is believed to be a higher energy requirement by the cell for readjustment of its intracellular composition to meet the new growth conditions.

Transient behavior in continuous culture has further been used for studying effects of inhibitors on growth and product formation, for studying mixed cultures and for elucidating dynamic elements in metabolic control.

DISCUSSION

Studies performed in some of the papers presented in this thesis (I, II, III, VI) and their applications are discussed below.

In large scale fermenters mixing is usually imperfect (7) and gradients of dissolved oxygen, pH, temperature and of the growth-limiting substrate in continuous culture may easily occur. The effects of transients on microorganisms are therefore important in the scaling-up and operation of fermentation processes.

In bench scale fermenters mixing is good and may be virtually perfect. Gradient formation of a growth-limiting nutrient in a large scale fermenter can be simulated in a bench scale fermenter by discontinuous feeding of all medium components, feeding of a concentrated solution of the limiting substrate or by shifts in the dilution rate.

When studying the effect of methanol gradients on the cell yield of Methylomonas methanolica WLO in a methanol-limited chemostat both *discontinuous feeding* and feeding of a concentrated methanol solution were used (I). The experimental set-up, which enabled us

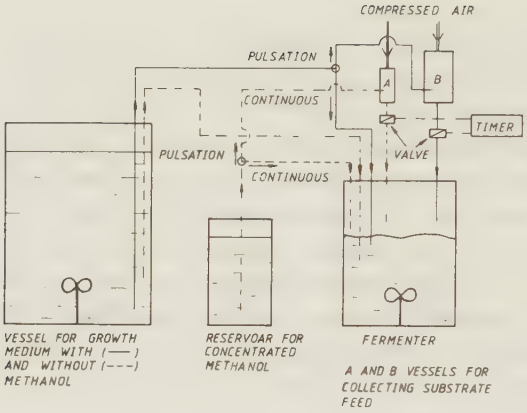


Figure 1. Diagram of experimental arrangement in a bench scale fermenter for simulating gradient formation in large scale fermenters.------- conditions where a separate concentrated methanol flow is used and ——— conditions where the main medium is containing methanol

to perform continuous and discontinuous feeding of a dilute as well as a concentrated methanol solution is shown in Fig. 1. These transient studies showed that methanol gradients markedly decreased the cell yield. In this case already the continuous feeding of a concentrated methanol solution caused gradient formation. When scaling up fermentation processes it is thus important to consider the problem of gradient formation when e.g. toxic substrates are used.

The utilization of mixed carbohydrates by a carbohydrate-limited culture of Streptococcus lactis 65.1 was also studied under transient conditions (II). In this case transients were induced by performing *shift-ups in dilution rate*. In S.lactis 65.1 maltose metabolism was subjected to catabolite repression and inhibition

mediated by glucose. Under steady-state carbohydrate-limiting conditions maltose and glucose were consumed simultaneously, whereas when the dilution rate was increased maltose utilization transiently stopped and maltose accumulated. Results from the transient studies showed that utilization of mixed carbohydrates in industrial scale carbohydrate-limited cultures may be incomplete when one of the carbohydrates (e.g. glucose) mediates catabolite repression and/or inhibition of the other component in the mixture. Gradients are apt to develop when a concentrated feed is used which is the case in e.g. microbial cultures with recycling of cells.

Investigation of the effects of transients on the metabolism of microorganisms might facilitate elucidation of the mechanisms involved in the intracellular control and regulation of growth and metabolism

Studies on transient responses following *dilution rate shifts* in a maltose-limited culture of S.lactis 65.1 suggested possible explanations of the rate-limiting step in the maltose metabolism in S.lactis (II). Similar transient studies performed in a maltose plus glucose-limited culture showed that it was the glucose uptake rate and not the glucose concentration that mediated the inhibition of maltose utilization.

Examination of the transient response to a *pulse addition* of methanol to a carbon-limited continuous culture of M.methanolica WLO at different dilution rates suggested regulatory mechanisms involved in causing the decreased cell yield, when the organism was exposed to methanol gradients during growth (I). Pulse additions were also used in the study of the regulation of glycolysis in S.mutans PR-89 and S.lactis 65.1. In the streptococci studied the lactate dehydrogenase (LDH), a key enzyme in the regulation of the pathway from hexose to lactic acid, was activated in vitro by fructose 1,6-diphosphate (FDP) and this activation was inhibited by inorganic phosphate. The transient responses of S.mutans PR-89 to pulse additions of glucose alone or glucose together with phosphate and/or the protein synthesis inhibitor

chloramphenicol (CAP) suggested that the organism was probably dependent on activation of LDH already present in the cell as well as de novo synthesis of LDH for its ability to increase the lactic acid production rate. Measurements of LDH activities during the transient period showed the occurrence of de novo synthesis of LDH (VI). Similar transients were induced in batch or continuous cultures of S.lactis on maltose (III). The transient responses showed that inorganic phosphate in vivo affected the specific lactic production rate as well as the pattern of product formation, information contributing to a better understanding of the mechanisms involved in the regulation of glycolysis when S.lactis 65.1 is grown on maltose (III).

In conclusion, transient conditions can be a problem in industrial fermentations but maintainance of the microorganisms under controlled transient conditions might be useful for increasing the yields of specific microbial products.

CELL YIELDS IN CULTIVATIONS OF *METHYLOMONAS METHANOLICA*

BACKGROUND

Methylotrophs have the ability to grow on carbon compounds containing no carbon-carbon bonds (one-carbon (C_1) compound). The growth of obligate methylotrophs is absolutely dependent on C_1-compounds, whereas the facultative methylotrophs can utilize other organic compounds for growth (2).

In all methylotrophs one-carbon substrates are assimilated into cell material at the formaldehyde level. Two main pathways for formaldehyde fixation are known viz. the ribulose monophosphate (RM) pathway or cycle and the serine (S) pathway. The fructose biphosphate (FBP) and the Entner Doudoroff (ED) pathways are two variants of the RM-cycle (Fig. 2) (69). Methylomonas methanolica WLO, which is an obligate methylotroph, reportedly incorporates formaldehyde via the ED variant of the RM-pathway (77, 52).

Methanol is oxidized to carbon dioxide either via formate or by a cyclic oxidation of formaldehyde (Fig. 3) providing NAD(P)H, ATP and carbon dioxide for growth (69, 3).

The P/O ratio is defined as the number of ATP molecules produced per atom of oxygen consumed, and values of 1 or 2 have been reported for the different steps of the methanol oxidation (74, 58). It has been suggested that in the facultative Pseudomonas AM1 the P/O ratio for substrates oxidized by NAD-linked dehydrogenases should be 2, whereas substrate oxidations not involving NAD should give P/O ratios of 1 (58).

The methanol dehydrogenase is not NAD-linked (3). Dehydrogenases which are NAD-linked and those which are not take part in the oxidation of formaldehyde to formate (2, 51, 60). In methylotrophs formate is oxidized by NAD-linked dehydrogenases (3). Two NADPH are formed in the cyclic oxidation of formaldehyde to carbon dioxide (69).

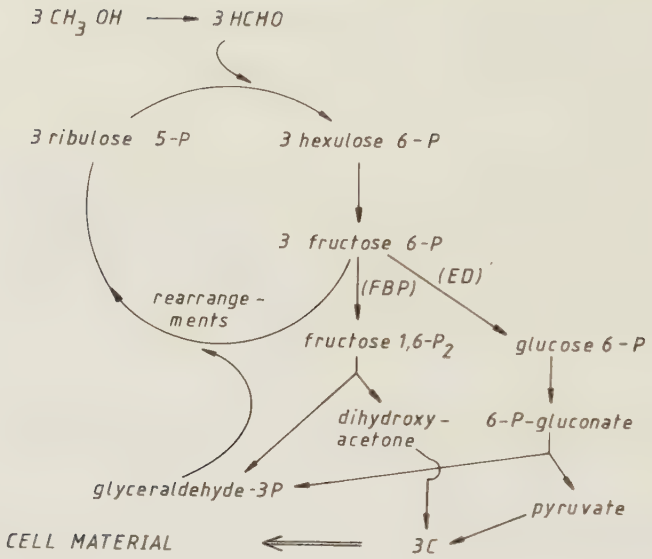


Figure 2. The ribulose monophosphate cycle of formaldehyde fixation

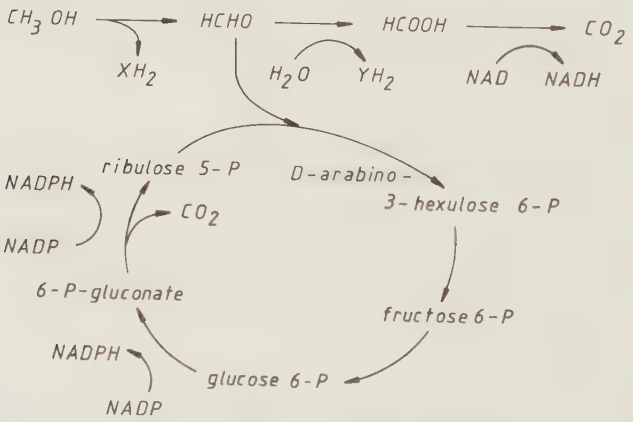


Figure 3. Alternative pathways for the oxidation of methanol in methylotrophs. XH_2 is reduced methanol dehydrogenase and YH_2 is the product of formaldehyde oxidation (sometimes NADH)

Anthony (3) used equations for the assimilation of methanol into cell material via the different pathways when predicting cell yields using various P/O ratios. In most methylotrophs the growth yield depends on the NAD(P)H as well as the ATP supply. For the ED variant of the RM-pathway the following equation for assimilation was developed assuming the formula $C_4H_8O_2N$ for cell material and Y_{ATP} (g cell/mole ATP) 10.5 for assimilation of phosphoglycerate.

$$12\ HCHO + 1.5\ NADH + 37\ ATP + 3\ NH_3 \longrightarrow 306\ g\ cell + 6\ H_2O$$

Cell yields in bacteria utilizing the RM-pathway are in general predicted to be higher than in cells incorporating formaldehyde via the serine pathway. Predicted yields using the same P/O ratios for organisms having the FBP variant of the RM-pathway are higher than those obtained for organisms utilizing the ED variant.

The efficiency of conversion of substrate carbon into cellular carbon is the most important parameter in a process for the production of single-cell protein (SCP) from methanol. A high conversion efficiency results in: (i) higher cell yield or yield coefficient (g dry weight/g substrate consumed), (ii) less oxygen being required per unit of cell produced, thereby lowering the costs for oxygen transfer, (iii) a decrease in the amount of heat evolved per unit cell produced, which means reduction of cooling costs. Protein yield is really the interesting yield, which is dependent on the cellular yield coefficient and the protein content of the organism.

SUMMARY AND DISCUSSION

The dependence of cell yield on environmental factors such as medium composition, pH, temperature, dissolved oxygen tension, carbon dioxide partial pressure and methanol concentration or distribution is discussed below.

Medium composition

It was shown that the medium used by Dostálek et al. (20) for the cultivation of M.methanolica did not support growth at high cell concentrations in continuous culture. Steady-state conditions could not be obtained at a cell concentration above approximatively 10 $g \cdot l^{-1}$.

The medium optimization technique used by Dostálek et al. (20) was based on a model where the medium components consumed by the organism would continuously be replaced by feeding a concentrated acidic (pH 2.0) nutrient solution (dosing solution). The composition of the starting medium, W8 (Table 1) and the dosing solution, W9 (Table 1) was based on the relations of individual elements in the biomass assuming that the proportion of minerals relative to nitrogen is constant in bacteria. Consumption of nitrogen was compensated for by addition of ammonium hydroxide to keep the pH constant. The dosing solution contained all other nutrients except nitrogen and was added at the same time as the dosing of ammonium hydroxide.

In a preliminary continuous culture experiment, where the concentration in the starting medium of calcium and most of the trace elements were substantially decreased, an increased cell yield and steady-state cell concentration was obtained. With the use of a starting medium containing, in $mg \cdot l^{-1}$: N 85, P 220, K 115, Mg 20, Fe 1.7, Ca 2.0, Cu 0.1, Zn 0.1, Mn 0.04, Co 0.04 (Mo and B were omitted) and the addition of the dosing solution, W9, continuous cultures were performed in the concentration range of 20 to 32 $g \cdot l^{-1}$ and the uptake of the elements K, Mg, P, N and Fe was measured. The results obtained are summarized in Table 2. The maximum uptake value for K, Mg, P and Fe with the addition of the following steady-state concentrations ($mg \cdot l^{-1}$): K 70, Fe 2.0, Mg 50, P 100 and N 300 were used for designing single stream media to support growth at different steady-state cell concentrations in continuous culture. The pH was controlled with the use of ammonium hydroxide, which continuously supplied the culture with nitrogen. The concentrations of calcium and trace elements (Cu, Zn, Mn, Co)

were kept constant at the values mentioned above and also indicated for MIA 3 (Table 1) and the single stream medium was tested up to a cell concentration of 40 $g \cdot l^{-1}$, where the oxygen transfer rate in the fermenter using air prevented a further increase. Additional supply of calcium and trace elements but keeping their steady-state concentrations well below those obtained when W8 and W9 dosing was used, did not affect the cell yield.

An analysis of composition of cells grown on MIA 3 or W8 with dosing of the solution W9 showed that the cell content of espeically calcium, iron, copper, and zinc varied with the growth medium used (Table 3). The concentration of these elements in the feed medium (Table 1) and in the fermentation broth at steady-state were markedly higher in the case of W8 and dosing of W9 compared with MIA 3. There was also a marked difference in the yield coefficients obtained, which strongly indicates that one or several of the elements mentioned were affecting cell yield. It should be noted that when a single stream medium was used at a steady-state cell concentration of 1.5 $g \cdot l^{-1}$ the yield coefficient was 0.53 (I). The carbon, nitrogen, hydrogen and oxygen content of M.methanolica WLO grown on MIA 3 (Table 1) agree with the formula, $C_4H_8O_2N$, used by Anthony (3) for cell material when calculating the theoretical yield.

These results showed that the proportion of minerals relative to nitrogen is not constant in bacteria, but dependent on the medium composition. When designing a medium to be used in a process for SCP production it is also important to use cell yield and not only specific growth rate (20) as a criterion.

Table 1. Composition of the dosing solution (W9), the single stream fermenter medium (MIA 3) and the starting medium (W8)

Element	W8 ($mg \cdot l^{-1}$)	MIA 3[1] ($mg \cdot l^{-1}$)	W9 ($mg \cdot l^{-1}$)
P	350	850	$5 \cdot 10^3$
K	35	270	500
Mg	28	150	400
Ca	21	2.0	300
Fe	14	9.0	524
Cu	1.1	0.10	16.4
Zn	1.1	0.10	0.016
Mn	0.14	0.040	2.0
Co	0.014	0.040	0.20
Mo	0.013	-	0.20
B	0.014	-	-
S	130	200	$1.85 \cdot 10^3$
N	400[2]	300	-

1) designed for a steady-state cell concentration of 27 $g \cdot l^{-1}$

2) the pH of the medium was adjusted to pH 6.5 with the use of NH_4OH before the cultivation was started

Table 2. Uptake of element by M.methanolica WLO in steady-state continuous culture at D = 0.13 - 0.15 h^{-1}, temperature 35° C and in the cell concentration range of 20 to 32 $g \cdot l^{-1}$

Element	mg/g cell dry weight		
K	4.7	-	7.1
Fe	0.20	-	0.28
Mg	2.6	-	3.8
P	22.7	-	27.3
N	120	-	103

Table 3. Cell composition and cell yield ($Y_{x/s}$) of M.methanolica WLO using different media in continuous culture at D = 0.12 - 0.17 h^{-1}

Medium[1]	X ($g \cdot l^{-1}$)	$Y_{x/s}$ ($g \cdot l^{-1}$)	% C	% N	% H	% O	mg·g DW^{-1} 2) K	Mg	P
W8+W9 dosing	5.0	0.30	49.9	13.7	7.52	24.5	6.75	3.13	27.7
MIA 3(single stream)	25.0	0.44	46.4	12.9	6.78	30.1	4.94	3.51	22.4

	mg·g DW^{-1} 2) Fe	Cu	Zn	Na	Ca	Co	Mn	Mo
W8+W9 dosing	3.51	0.141	0.310	ND	2.40	0.0028	0.042	0.040
MIA 3(single stream	0.332	0.005	0.006	7.1	0.033	<0.002	0.006	0.009

1) for explanation see text
ND not determined
2) DW = dry weight of cells

Methanol

The specific growth rate of M.methanolica decreases with increasing methanol concentration above a critical value of around 3.0 $g \cdot l^{-1}$ (substrate inhibition) (21). A concentrated methanol solution was fed to the culture throughout the batch fermentations in order to maintain the methanol concentration in the broth below 3.0 $g \cdot l^{-1}$.

The cell yield values observed in batch cultures were lower than in corresponding methanol-limited chemostat cultures and varied between 0.25 and 0.40 depending on the medium used (see "medium composition" above. In methanol-limited chemostats the yield

coefficient varied with the concentration of methanol in the medium feed. Locally high methanol concentrations resulted in a lower yield coefficient for methanol (I). The low cell yields observed in the batch cultures were therefore probably due to gradient formation caused by the addition of concentrated methanol solution.

It was found that formate transiently accumulated after a pulse addition of methanol to a continuous culture (I). A pH increase observed at the end of a batch culture may be due to formation of formate or possibly also to degassing of carbon dioxide. The increase in yield coefficient when a culture was exposed to locally high methanol concentrations may thus partly be explained by formate formation which probably also leads to NAD(P)H-limited growth. Both these factors increase the requirement of methanol for energy production per g cell produced. As pointed out by Brookes and Meers (6), the lowered cell yield may be partly due to energy wasting when the organism is constantly changing its growth rate. Hypothetically, methanol itself may also decrease the phosphorylation efficiency. Ethanol has been reported to have this effect on the yeast Torulopsis utilis (33), and in the bacterium Klebsiella aerogenes (83) the specific respiration rate increased in the presence of ethanol. Puhar et al. reported (62) that high methanol concentrations in the medium feed, and a shift in continuous culture to a spent medium from a chemostat performed at a high cell concentration, decreased the potential oxygen specific uptake rate (q_{O_2}) of M.clara. These results strongly indicate that a excreted compound, possibly formate, is causing the decrease in q_{O_2}. Formate is reported to inhibit the cytochrome c oxidase, which is involved in the oxidation of methanol in methylotrophs (31).

The maximum yield coefficient 0.52 - 0.53 $g \cdot l^{-1}$ obtained with M.methanolica WLO agrees well with the ED variant of the RM cycle and the P/O ratios 1,1 and 2, respectively for XH_2, YH_2, NADH (Fig. 2) which (3) give a predicted yield of 0.50 . M.methanolica does not appear to have the cyclic oxidation of formaldehyde (I) (Fig. 3). These results indicate that the only NADH-yielding step

in the energy metabolism of M.methanolica WLO is the conversion of formate to carbon dioxide, which increases the risk of NADH-limitation if formate is excreted.

Other environmental factors

Low *dissolved oxygen tensions* (DOT) decreased the cell yield of M.methanolica WLO, and the critical DOT below which cell yield started to decrease was estimated (I) (Fig. 4). The critical DOT obtained differed from what was expected from the literature. The electrode measuring the DOT did, however, register the DOT in the bulk of the liquid and not the oxygen concentration experienced by the cell and its cytochrome system. The apparent critical DOT therefore most likely varies with growth conditions, such as cell concentration (I). An increased specific respiration rate has been reported in Pseudomonas AM1 (45) and K.aerogenes (28) at low DOT indicating an uncoupling of oxidative phosphorylation.

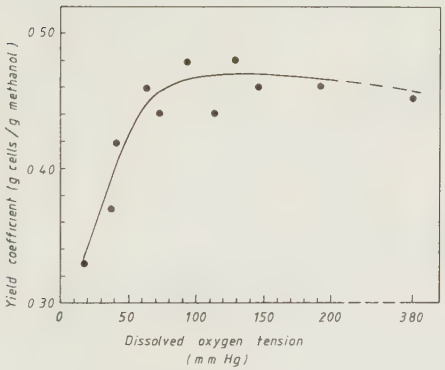


Figure 4. Yield coefficient as a function of dissolved oxygen tension in M.methanolica WLO

The partial pressure of *carbon dioxide* decreased the cell yield of M.methanolica WLO above 0.30 atm, which may occur in high fermentation vessels or if oxygen is used instead of air.

On change of the *pH* from 6.5 to 6.0 there was a 12 % decrease in cell yield, and an increase in pH from 6.5 to 6.7 caused a 6 %

decrease of the yield coefficient in continuous culture. These results indicate that also pH gradients may decrease the cell yield. An effect of the acidic dosing solution (see "medium composition" above) used in the earlier work can therefore not be ruled out.

A change in the steady-state growth *temperature* in the chemostat from 35 to 30° C resulted in a 8 to 10 % decrease in cell yield.

In the *dilution rate* range of 0.10 to 0.30 h^{-1} the yield coefficient for methanol was practically constant. The measurements were made in the cell concentration range of 8 to 24 $g \cdot l^{-1}$ and at approximately the same cell productivity. As the yield was shown to be dependent on methanol gradients in the broth, the yield coefficient observed probably depends on the cell concentration and the productivity, which are proportional to the concentration of methanol in the medium feed and the rate of addition of methanol, respectively. Determination of yield dependence on dilution rate should therefore be carried out at low steady-state cell concentrations.

PRODUCT YIELDS IN CULTIVATIONS OF LACTIC ACID BACTERIA

BACKGROUND

Streptococci and pediococci ferment hexoses via the Emden-Meyerhof-Parnas (EMP) pathway and are regarded as homofermentative and the main product of the energy metabolism is lactic acid (18). Heterofermentative product formation may, however, occur in streptococci and then not only lactate, but also acetate, ethanol, formate as well as carbon dioxide may be formed (71, 81). Fig. 5 gives a diagram of the different possible catabolic pathways.

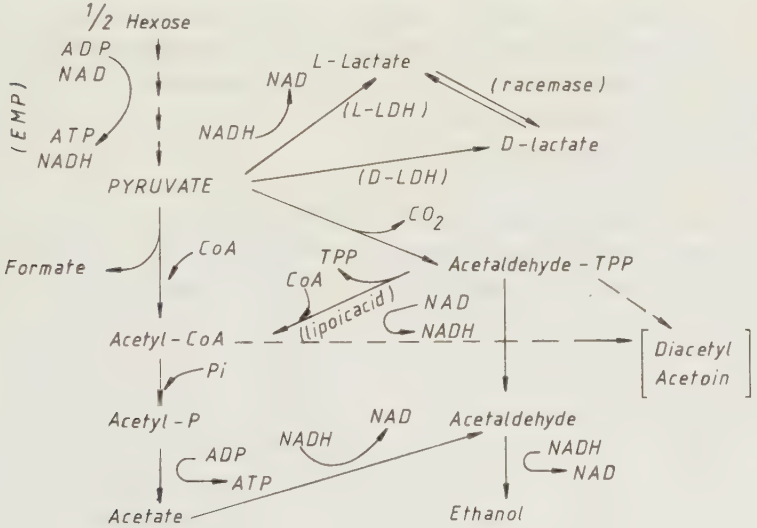


Figure 5. Different catabolic pathways present in lactic acid bacteria metabolizing hexoses via the EMP pathway

In most lactic acid bacteria L(+) and D(-)-lactic acid is formed via the stereospecific L- and D-lactate dehydrogenases (L-LDH and D-LDH). Some organisms may form DL-lactic acid by using a racemase and probably a L-LDH (38, 22). Streptococci reportedly form

L-lactic acid via a NAD-dependent L-LDH (18, 38, 22, 1). Pediococci produce D- and L-lactic acid (38, 24). N-streptococci can produce diacetyl and acetoin via the acetaldehyde/TPP complex and the formation of carbon dioxide (18, 10). Streptococci can also use the alternative route from pyruvate to acetate via a pyruvate formate-lyase (71, 81, 43).

L-lactate dehydrogenases (L-LDH) from lactic acid bacteria may be of two different kinds, those which are activated by fructose 1,6-diphosphate (FDP) and those which are not (22). The L-LDH from S.cremoris (32, 22) S.lactis (16, 22) and S.mutans (81, 22) and the pyruvate kinase from S.lactis (15, 11) are both activated by the glycolytic intermediate fructose 1,6-diphosphate (FDP) in vitro. The activated enzymes are both inhibited by orthophosphate. These two allosteric enzymes are involved in the conversion of phosphoenolpyruvate to lactic acid and are therefore important regulatory enzymes in the glycolytic pathway of streptococci. D-LDH is not activated by FDP (22).

The membrane-associated phosphoenolpyruvate (PEP) dependent phosphotransferase system (PTS) for carbohydrate transport is reported to exist in streptococci, pediococci and other anaerobes in which sugars are metabolized via the EMP-pathway (21, 64, 25).

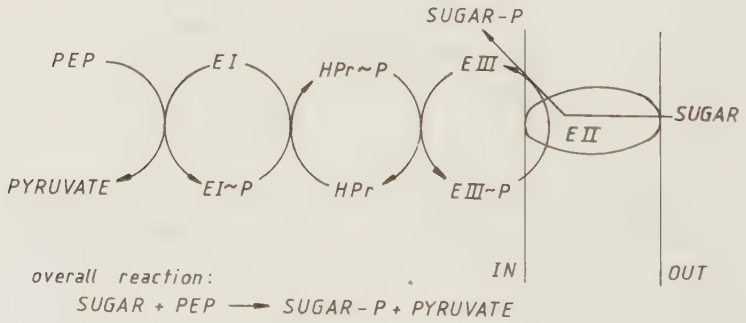


Figure 6. Phosphoenolpyruvate sugar phosphotransferase system. EI is enzyme I, EII is enzyme II, EIII is enzyme III, HPr is a phosphocarrier protein and~P denotes a phosphorylated compound

In the PEP sugar PTS (Fig. 6) enzyme I and the protein HPr are general proteins, whereas enzyme III, if present and enzyme II are usually specific for the sugar (19, 79). In S.lactis lactose and glucose are reportedly transported via PTS, whereas galactose can be transported either by the PTS or a permease (72). Sucrose, lactose and glucose are phosphorylated and transported by the PTS in S.mutans (25).

Carbohydrate metabolism and uptake in bacteria may be regulated in different ways. The main mechanisms involved in regulating carbohydrate utilization are; (i) induction, (ii) catabolite repression, (iii) transient repression and (iv) catabolite inhibition. For the synthesis of an inducible enzyme the presence of an inducer is necessary but often not sufficient. Catabolite repression reduces the rate of synthesis of specific enzymes when glucose or another readily metabolized carbon source is rapidly catabolized (46). Transient repression may occur following addition of a new carbon compound, which is transported by the PEP dependent PTS (46). Catabolite inhibition is a glucose mediated inhibition of the utilization of another carbohydrate and is regulating the enzyme acitivty. Active transport of glucose across the cell membrane causes the catabolite inhibition (49).

For the control of carbohydrate uptake five mechanisms involved in causing inhibition have been suggested; (i) competition between two sugars for the binding of permease protein, (ii) PTS mediated regulation of non-PTS sugar permease activity, (iii) intracellular sugar phosphates, (iv) chemiosmotic control, (v) competition of two enzyme II sugar-complexes for the common HPr$\sim$P (Fig. 6) (19). There is evidence that the regulatory protein involved in the PTS-mediated regulation ((ii) above) of non-PTS sugars is the enzyme III^{glu} (Fig. 6). This regulatory system is itself regulated by "desensitization" which is induced by cyclic-AMP. Later studies have revealed that catabolite repression of non-PTS carbohydrates catabolic enzyme synthesis by a PTS carbohydrate may be due primarily to inducer exclusion by inhibition of inducer uptake. Cyclic-AMP abolishes inducer exclusion by inducing desensitization of the permease (19).

SUMMARY AND DISCUSSION

This section is focused mainly on factors influencing the relative product yields and the regulation involved on the metabolic level in the lactic acid bacterium Streptococcus lactis 65.1. A comparison is made between S.lactis 65.1 and two other organisms, a strain of Pediococcus pentosaceus and S.mutans PR-89, which belong to the same family as S.lactis. The discussion is based on papers II to VI.

Product yields

The choice of metabolic pathway was influenced by cultivation conditions, such as:

continuous or batch operation, and
carbohydrate source

When the various organisms were grown in batch culture on glucose only lactic acid formed (II, IV, VI). S.lactis and S.mutans produced the L-isomer and P.pentosaceus formed the L- and, though in small amount the D-isomer of lactic acid. P.pentosaceus and S.mutans showed the same homofermentative character on sucrose, which was not metabolized by this strain of S.lactis. Heterofermentative product formation occurred in glucose-limited chemostats of S.lactis (III) and S.mutans (VI). Not only lactic acid, but also acetic acid, ethanol and formic acid or carbon dioxide were produced. The contribution of the two different pathways (Fig. 5) to the heterolactic fermentation can be estimated from the molar ratio of formate to acetate plus ethanol. In S.lactis 65.1 (II, III) and S.mutans PR-89 (VI) the pathway from pyruvate with the formation of formic acid was dominant. The heterofermentative character increased with decreasing dilution rate. Similar results were obtained in lactose-limited cultures of S.lactis. These results were comparable to those found by Yamada and Carlsson (81) and Thomas et al. (71). Judging from an experiment in which the growth rate of S.lactis was lowered by decreasing the temperature the growth rate per se did not affect

the choice of pathway. P.pentosaceus grown in sucrose-limited continuous culture did not change to heterofermentative product formation but the ratio of D- to L-lactic acid increased with decreasing dilution rate. Small amounts of acetic acid were produced at low growth rates, probably by way of decarboxylation of pyruvate since no formic acid was detected. A pulse addition of glucose to the glucose-limited culture of S.mutans PR-89 (VI) as well as to S.lactis 65.1 resulted in an increased lactate yield corresponding to 90 - 100 % of the theoretical and a specific lactic acid production rate, which was some times higher than the maximum rate observed in batch culture.

The nature of the carbon source influenced the yield of products. S.lactis 65.1 (II) in batch culture showed a lower lactate yield on galactose than on glucose and lactose. Maltose metabolism in S.lactis (II) resulted in a pronounced heterofermentative product formation with lactate yields around 40 % in batch culture. In a continuous maltose-limited chemostat of S.lactis (III) product yields were almost constant over the whole dilution rate range but did not differ much from the yields obtained in batch culture on maltose. Product yields from mixed carbohydrates (galactose + glucose + lactose and glucose + maltose) in batch culture varied over the growth period (I) and diauxic growth was noted. In continuous culture maltose and glucose were metabolized simultaneously and the product yields showed the same dependence on dilution rate as in the glucose-limited culture, but the heterofermentative character at each dilution rate was between that observed on the individual sugars.

Carbohydrate utilization and regulation

Sequential utilization of sugars occurred in S.lactis 65.1 (II), and galactose or maltose were not metabolized in the presence of glucose. Lactose and glucose were utilized simultaneously. In P.pentosaceus glucose and sucrose were metabolized at the same time.

Catabolite inhibition and repression were involved in the control of maltose metabolism in S.lactis (II). Similar control mechanisms appear to be involved in the utilization of galactose in this organism. Transient responses in continuous culture showed that it was the high glucose uptake rates and not the glucose concentration as such that inhibited the uptake of maltose. An inducible maltose hydrolyzing enzyme, which was dependent on orthophosphate for activity was found in S.lactis 65.1 (II, III). The maltose phosphorylase was under catabolite repression control mediated by glucose, and the maltose phosphorylase level varied with the flow of maltose through the cell at a constant extracellular maltose concentration.

Permeabilized cells of S.lactis 65.1 phosphorylated glucose, galactose and maltose in the presence of phosphoenolpyruvate (PEP). As for maltose, there was most likely an artefact. As permeabilized cells were used the maltose molecule is probably cleaved after which the glucose moiety is phosphorylated by PEP. It appears likely that the maltose phosphorylase is specific for maltose and that maltose is transported by a permease into the cell where it is cleaved and phosphorylated. The hydrolytic enzymes β-galactosidase and β-phosphogalactosidase in N-streptococci were e.g. shown to be substrate specific (57). Galactose has also been reported to be transported via a permease and then phosphorylated in the cell (72).

Maltose was hydrolyzed to glucose and β-glucose 1-phosphate (β-glc-1P) by cell-free extracts of S.lactis 65.1. The fate of β-glc-1P in the cell is not clear. The following pathways were ruled out:

(i) β-glc-1P $\rightarrow$ α-glc-1P as no NADP reduction was observed in the reaction.

$$\beta\text{-glc-1P} \xrightarrow[\text{extract}]{\text{cell-free}} \alpha\text{-glc-1P} \xrightarrow[\text{glucomutase}]{\text{phospho-}} \alpha\text{-glucose 6-P}$$

$$+ \text{NADP} \xrightarrow[\text{dehydrogenase}]{\text{glucose 6-P}} \text{6P-gluconolactone} + \text{NADPH}$$

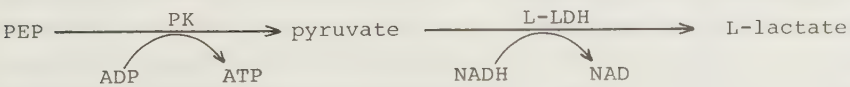
(ii) β-glc-1P + ATP $\longrightarrow$ fructose 1,6-diphosphate

as no NADH oxidation was observed when NAD, aldolase, triose-phosphate isomerase and glycerol-3P dehydrogenase were added to the assay system. As glucose-1P was identified in the reaction mixture when cell-free extract reacted with maltose (III) it appears likely that energy in som form is necessary for the intracellular conversion of β-glc-1P before it can enter the glycolytic pathway. Hypothetically, β-glc-1P is converted to UDP-β-glucose by UTP and a pyrophosphorylase and then yielding the α-anomer of glucose 1-P. This conversion was not obtained with GTP, but UTP remains to be tried.

In N-streptococci there are two pathways for galactose metabolism (73); the D-tagatose-6-phosphate pathway operating when galactose is transported via the PEP-sugar-PTS and the Leloir pathway operating when galactose is transported via a permease and phosphorylated intracellularily by ATP. In the Leloir pathway galactose-1P is converted to glucose-1P via UDP-glucose and UDP-galactose. In Bifidobacterium bifidum (41) an alternative route from galactose 1-P to glucose 1-P was found; it involved a UDP-glucose and a UDP-galactose pyrophosphorylase, UTP and pyro-phosphate.

Regulation of glycolytic pathway and product formation

Key regulatory enzymes in the glycolytic pathway from hexose to pyruvate in streptococci are L-lactate dehydrogenase (L-LDH) and pyruvate kinase (PK). These enzymes catalyze the following reactions;



The L-LDH activity of the cell determines the specific lactic acid production rate, and the activity of PK most probably affect

both the intracellular PEP and pyruvate concentrations. As mentioned earlier, PEP takes part in the transport of some sugars.

The enzyme activity of the cell may be regulated in two ways, namely, by change in activity of existing enzyme and de novo synthesis.

L-lactate dehydrogenase from streptococci are reportedly activated by the earlier intermediate in the glycolysis fructose 1,6-diphosphate (FDP), and the FDP-activated L-LDH is inactivated by orthophosphate (81, 16, 32). The L-LDH from S.lactis 65.1 was shown to be of this kind and in addition to orthophosphate, pyrophosphate was shown to inhibit the activated L-LDH (III). In streptococci there are thus besides NADH and pyruvate concentration at least two other regulators of the L-lactate dehydrogenase activity, namely, FDP and inorganic phosphate. Pyruvate kinase from N-streptococci was found to be regulated in the same way as the L-LDH, but the FDP activated enzyme appeared to be more sensitive to inhibition by inorganic phosphate (15, 11). P.pentosaceus possessed a L- and a D-lactate dehydrogenase, and no activation by FDP was observed.

Heterofermentative product formation in glucose-limited continuous cultures of S.mutans has been explained by a decreased intracellular FDP concentration resulting in a decreased activity of existing LDH (81). It was, however, shown that S.mutans PR-89 controlled the L-LDH activity per cell by activation of existing L-LDH as well as de novo synthesis (VI). In S.lactis 65.1 the L-LDH activity of the cell was shown to increase at the higher dilution rates in a glucose-limited culture (III), which agrees with the results obtained by Thomas et al. (71). Thus, in streptococci the LDH level can very and LDH activity is affected by the intracellular FDP concentration.

In the regulation of the D- and the L-lactate dehydrogenase in P.pentosaceus it appears as if it is the L-LDH level that regulates the metabolic flow to L- and D-lactate. In batch culture the yield of the two lactic acid isomers was proportional to the

activity of the two lactate dehydrogenases (IV).

Measurements of LDH and FDP in batch and LDH in continuous cultures where S.lactis 65.1 was growing on different sugars clearly showed that there must be additional regulatory factors involved in control of product formation.

Both S.lactis (III) and S.mutans PR-89 showed a transient decrease in specific lactic acid production when they were suddenly exposed to increased orthophosphate concentrations in vivo. In S.mutans the decrease in specific lactic acid production increased with the amount of phosphate added (VI). In S.lactis a transient switch in metabolism towards higher ethanol and acetic acid yield was observed when orthophosphate was added to a batch culture growing on maltose.

As mentioned above, S.lactis 65.1 induces a maltose phosphorylase when grown on maltose (II, III). Steady-state and transient experiments in maltose-limited continuous cultures showed that inorganic phosphate probably did not limit the maltose hydrolyzing rate in vivo (III). Thus the intracellular phosphate concentration was presumably above 50 mM. It was therefore suggested that in cells growing on maltose the intracellular orthophosphate concentration plays an important role in regulating L-LDH and probably also pyruvate kinase, resulting in the formation of less lactic acid and more ethanol and acetic acid. In the galactose metabolism pyrophosphate which was shown to affect LDH activity, may be involved as indicated above.

In addition to the direct regulatory function of orthophosphate, involved in the maltose phosphorylase reaction on the L-LDH and pyruvate kinase activities, the need for transport of phosphate may affect the product formation. Harold et al. (27) investigated phosphate uptake by S.faecalis and proposed that accumulation of phosphate required ATP. Unexpected results were obtained when S.lactis 65.1 was grown in a maltose-limited culture at different steady-state phosphate concentrations (5 mM and 100 mM) (III). At the low phosphate concentration the lactate yield was lower than

at the higher phosphate level. These results indicate that the intracellular phosphate concentration may vary and/or that ATP is necessary for transport of phosphate at the lower, but not at the higher, phosphate concentration. When acetate is formed from pyruvate, ATP is obtained (Fig. 5) and the intracellular phosphate concentration may vary and be involved in regulating the pathway towards acetate formation when ATP is required.

Application to a mixed culture

Streptococci and pediococci are used as starter cultures for fermented food products. Fermentation of high-starch raw materials by these lactic acid bacteria is possible if another organism hydrolyzes the starch to fermentable sugars. The product yields of S.lactis 65.1 obtained under different growth conditions (II, III) were compared with the performance of S.lactis in a mixed culture where the starch hydrolyzing organism was the aerobic Saccharomycopsis fibuliger Y76 (V). The main interactions in the mixed culture were commensalism and competition. Continuous mixed cultures where organisms are competing for the same substrate are unstable. Two or several limitations may give ranges of dilution rates where the steady-state is stable. An external limitation was therefore applied to the mixed cultures of S.lactis and S.fibuliger. S.fibuliger accumulated sugars under oxygen-limiting conditions, for which reason oxygen was chosen as the regulatory parameter. Depending on the growth conditions different relative and total product yields were observed. The results could be explained by the difference in fermentation pattern of S.lactis when grown on respectively maltose and glucose. Product formation occurring in batch culture showed that maltose and glucose were fermented simultaneously. The level and the production rate of glucose during the fermentation must therefore be low. These results showed that when choosing lactic acid starter culture for raw materials other than the conventional ones, it is necessary to know how the source of carbon will affect the product formation in order to e.g. avoid unexpected product formation (Fig. 7).

Figure 7. A mixed culture of S.lactis and S.fibuliger

CONCLUSIONS

Parameters influencing cell and product yields in bacterial cultivations have been identified. In order to get a better understanding of the in vivo observed behaviour and to make the results more generally applicable mechanisms involved on the metabolic level were studied.

The *cell yield* in Methylomonas methanolica WLO was affected by medium composition, temperature, pH, dissolved oxygen tension, carbon dioxide partial pressure and locally high methanol concentrations in the fermentation broth. The decreased cell yield when the organism was exposed to locally high methanol concentrations could partly be explained by a non-optimally working energy metabolism and a NADH-limited growth due to the excretion of formate. It was shown to be important to use cell yield as a criterion when designing a medium to be used in a process for single-cell protein (SCP) production. The proportion of elements relative to nitrogen were shown to vary with the medium composition in M. methanolica. In order to maintain a high cell yield when scaling up a process for the production of SCP from methanol the formation of especially methanol and dissolved oxygen gradients have to be minimized in the large scale fermenter.

Continuous or batch operation and the nature of the carbohydrate source influenced *product yields* in streptococci (Streptococcus lactis and S. mutans) and to some extent in the pediococcus Pediococcus pentosaceus.

In the lactic acid streptococci the specific lactic acid production rate was regulated by the L-LDH activity as well as the de novo synthesis of L-LDH. The L-LDH level in streptococci may thus vary and the L-LDH activity is increased in the presence of fructose 1,6-diphosphate (FDP). Orthophosphate influenced the product formation in vivo and the L-LDH activity in vitro. The intracellular

phosphate concentration was suggested to be the main regulator of product formation when S. lactis was grown on maltose.

An inducable maltose phosphorylase which was subjected to catabolite repression mediated by glucose was found in S. lactis 65.1. Phosphorylases may be of potential interest for the synthesis of di- or tri- saccharides as the equilibrium may be controlled by the phosphate concentration in the reaction mixture.

An understanding of the regulation of product formation in the primary metabolism of fermentative bacteria is of significance for the possible use of these organisms in the production of chemicals and energy compounds from carbohydrate containing substrates. It is important to either choose the right process technology and raw material for the production of specific metabolic product(s) or to choose the right organism(s) in a culture to be used for a specific raw material.

The substrate availibility of S. lactis was extended to include starch by using a mixed culture where the other organism in the mixture was Saccharomycopsis fibuliger. The composition of the culture was stabilized by the external regulator oxygen. This kind of regulation may be applied to mixed cultures where one organism is dependent on oxygen for growth and the other organism is aerotolerant. The use of mixed cultures may be an alternative or a complement to genetic modifications of organisms in systems where complex substrates such as starch or cellulose is one of the raw materials.

On order to avoid gradient formation in large scale fermentation processes, where feeding of a mixed or toxic substrate is used, the distribution or mixing may have to be designed using other criteria than only the usual one; the distribution of oxygen. Regular pulse feeding may, however, be used to enhance overall product yields in fermentation processes especially when the metabolite to be produced is a product of energy metabolism.

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Growth of Methylomonas methanolica:
Factors influencing growth yield.

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SUMMARY

In a process for biomass production from methanol using Methylomonas methanolica WLO the efficiency of carbon conversion into cellular carbon is affected by external factors as:

(i) Dissolved oxygen tension (DOT) and carbon dioxide partial pressure (P_{CO_2}). At P_{CO_2} > 0.3 atm the cell yield is decreasing. Data suggests that "critical" DOT is dependent on cell concentration.

(ii) Methanol gradients. When supplying methanol discontinuously or in a concentrated feed a substantial drop in cell yield is obtained. Formic acid transiently accumulates following a pulse addition of methanol. As a result more methanol is needed for energy production and a lower cell yield is obtained. In addition a non-optimally working energy metabolism most likely leads to NADH limitation of cell synthesis.

INTRODUCTION

In a fermentation process for the production of single-cell protein (SCP) from methanol the yield coefficient for biomass formation from the carbon source (g biomass per g methanol consumed) is an economically critical parameter.

The two main pathways by which bacteria growing on methanol can incorporate C_1-units into cell material are the ribulosemonophosphate (RM) pathway and the serin (S) pathway (Anthony, 1975). The fixation of carbon into cell material is done at the level of formaldehyde. The general pathway for oxidation of methanol is via formaldehyde and formate to carbon dioxide (Anthony, 1978). A cyclic oxidation of formaldehyde to carbon dioxide has been reported in some methylotrophs (Strøm et al 1974). According to theoretical calculations of cell yield, organisms using the RM-pathway generally show higher efficiency in converting substrate carbon into cellular carbon (Anthony, 1978).

Specific growth rate and environmental conditions such as temperature, pH and medium composition have been shown to influence the yield coefficient for biomass formation from methanol (Dostálek et al, 1972; Dostálek et al, 1975; Häggström, unpublished data). Parameters such as carbon dioxide partial pressure, dissolved oxygen tension and the distribution of the toxic substrate methanol can be expected to be of great importance in the scaling up of this particular process. In large scale fermenters concentration gradients may occur due to e.g. insufficient mixing.

Studies reporting on the effect of carbon dioxide partial pressure on microbial growth have mostly dealt with its effect on specific growth rate. In Saccharomyces cerevisiae 50 % carbon dioxide in the aeration mixture caused a 16 % decrease in cell yield (Chen and Gutmanis, 1976). Inhibition of decarboxylating enzymes by carbon dioxide has been shown in in vitro experiments (King and Nagel, 1975). There are several reports on the influence of dissolved oxygen on microorganisms (Harrison, 1972, Harrison, 1976). When Pseudomonas AM1 was exposed to low and high dissolved oxygen tensions an increase in respiratory activity occured and at the same time a decrease in cell yield was obtained (MacLennan et al, 1971).

Transient phenomena in continuous culture have been extensively reviewed (Harrison and Topiwala, 1974; Cooney et al, 1981). The effect of dicontinuous feeding of the growth limiting substrate to a chemostat have been described for bacteria (Brooks and Meers, 1973; Luscombe, 1974; Nagai, 1968) and for yeast (Welles, 1976; Vairo, 1977). Discontinuous feeding of the substrate to a methanol-limited culture of Pseudomonas methylotropha resulted in a decrease in biomass yield. In chemostat cultures of S. cerevisiae higher ethanol yields were obtained under conditions of discontinuous substrate feed.

In this paper we report on the effect of carbon dioxide, dissolved oxygen and methanol gradients on the cell yield of Methylomonas methanolica WLO. This study was made with reference to scaling-up problems especially at high cell concentrations. In order to simulate gradient formation transient conditions were induced in laboratory fermenters. In addition we report on the mechanisms involved on the metabolic level.

MATERIAL AND METHODS

Organism. Strain WLO of Methylomonas methanolica was isolated in a cultivation of M. methanolica NRRL B-5458. Both strains use the ribulose monophosphate cycle for formaldehyde fixation (Undén, 1977).

Growth medium. The medium used for maintaining the culture and for growing inocula contained ($g \cdot l^{-1}$) NH_4Cl 0.38, $Na_2HPO_4 2H_2O$ 0.24, NaH_2PO_4 1.2, Na_2SO_4 0.45, KCl 0.17, $MgCl_2 6H_2O$ 0.33, Na-citrate 0.10 (to avoid precipitation), methanol 4.0 and ($mg \cdot l^{-1}$) $CaCl_2 2H_2O$ 4.0, $ZnSO_4 7H_2O$ 0.22,

$CuCl_2 2H_2O$ 0.13, $MnSO_4 H_2O$ 0.06, $CoCl_2 6H_2O$ 0.08, $FeCl_3 6H_2O$ 12.0. Urea (0.6 $g \cdot l^{-1}$) was added to the shake flask cultures. The medium used for fermenter cultivations was the same but without urea and to support growth at the cell concentration desired the different amounts were adjusted according to data on uptake of the different elements (Häggström, unpublished data). The pH was controlled by the addition of NH_4OH which continuously supported the culture with nitrogen.

Cultivation equipment and conditions. The batch and continuous cultivations were performed in a fermenter (Chemoferm AB, Hägersten, Sweden) with a working volume of 4 litre. Temperature and pH were controlled at 6.5 and 35°C respectively. Dissolved oxygen tension in the broth was measured by a galvanic probe (Johnson et al, 1964). During the batch cultures the methanol concentration in the gas phase was continuously measured and the concentration in the broth was controlled at 2.4 g/l. The amounts of ammonia and methanol consumed were continuously registered using two monitors (Enfors and Dostálek, 1975). In the continuous culture experiments methanol was the growth limiting substrate (chemostat). The cultivation volume was kept constant using a conductivity type level regulator. Foaming was controlled in a similar way but with the addition of the antifoam Adekanol LG-109 (Asahi Electro Chemical Co, Japan).

The culture was taken to be at steady-state when cell concentration, dissolved oxygen tension and ammonia consumption rate had been constant for a period of at least three times the actual residence time. Where not otherwise stated the experiments were carried out in the dilution rate range of 0.12-0.18 h^{-1}. The cell yield is constant in this range of dilution rates (Häggström, unpublished data).

Growth measurement. Optical density of the cell suspension was measured in a Turner photometer at 620 nm. Cell dry weight was measured by filtering a defined volume of cell suspension through a predried 0.45 µm membrane filter (Millipore Bedford, Mass, USA). The filter was dried for 1 hour at 105°C and weighed.

Analyses. Methanol concentration in the medium and in the supernatants was measured by gas chromotography on a 1/8 inch I.D., 5 ft long stainless steel Porapak Q column using a Varian 940 gas chromatograph equipped with a hydrogen flame ionization detector. The column temperature was

130°C and nitrogen was used as carrier gas at a flow rate of 30 ml/min. When measuring methanol in the gas phase the gas flow was continuously fed to the detector of the chromatograph.

Oxygen and carbon dioxide in the gas flow were measured periodically. Samples were injected into a Varian 920 gas chromatograph equipped with a thermal conductivity detector and two 1/8 inch I.D., 6 ft stainless steel columns in a series. One Porapak Q column was kept at 90°C for the separation of carbon dioxide and the other column with Molecular Sieve 5A was kept at room temperature for the separation of oxygen and nitrogen. The second column was inserted between the sample and the reference detector. For calibration a standard gas mixture (Alfax, Malmö, Sweden) was used. In some experiments carbon dioxide was measured continuously using an infrared carbon dioxide analyzer (Binos 1, Leybold-Hereus, Köln, Germany).

Formate was determined according to Lang using citric acid and the absorbance was measured at 515 nm (Lang and Lang, 1972).

Carbon dioxide and dissolved oxygen experiments. In the continuous culture the carbon dioxide level and the dissolved oxygen partial pressure in the fermentation broth were varied by mixing carbon dioxide and oxygen into the air stream. Carbon dioxide was measured in the exhaust gas. This value is a direct measurement of the partial pressure of dissolved carbon dioxide in a non-viscous medium (Alford, 1976).

The response from the galvanic electrode used for measuring dissolved oxygen was calibrated in dissolved oxygen tension (DOT), which is the partial pressure of oxygen in the gas phase in equilibrium with the fermentation broth. There was a linear relationship between the output signal from the oxygen electrode and the DOT in the range used. In the dissolved oxygen experiments the stirrer speed and the gas flow rate were kept constant during each set of experiments.

Discontinuous methanol feed. The discontinuous feeding of methanol to a chemostat culture was carried out in two different ways.
(i) Methanol was discontinuously fed to the culture in a separate stream while the other medium components were fed continuously. This was performed by collecting the methanol flow in a reservoar, the contents of which was added to the fermenter at different intervals. The time between the additions is called pulse time. By using this arrangement the flow of methanol could be measured and kept constant.

(ii) Methanol was mixed with the other medium components and the whole flow was fed to the fermenter at different intervals. This was carried out in the same way as described above but this time the whole medium flow was collected in the reservoar before being added to the fermenter.

In both cases described above the measurements were carried out when the culture had been subject to the pulsation procedure for at least three times the actual residence time.

Methanol pulse experiments. A methanol pulse was added to a chemostat culture at steady-state. The amount added was adjusted to give an initial concentration of 4 $g \cdot l^{-1}$ in the broth. This methanol concentration does not affect the maximum specific growth rate of the organism. During the transient period that followed, samples were taken every 15-30 minutes for determining residual methanol and cell concentrations. The partial pressure of carbon dioxide in the outgoing gas stream was continuously registered during the transient period.

Calculations. In the continuous culture experiments at steady-state and during conditions of discontinuous feed the yield coefficient for biomass formation from methanol was determined using the relationship

$$Y = \frac{X}{S_o - S}$$

The data obtained during the transient period following a pulse addition of methanol were used for calculating the following parameters for a small interval $\Delta t = t_2 - t_1$

yield of biomass $(Y_{x/s}) = \frac{\Delta X}{\Delta S}$ $(g \cdot g^{-1})$

specific CO_2 production $= \frac{\Delta CO_2}{\Delta t} \cdot \frac{1}{X}$ $(g \cdot h^{-1} \cdot g^{-1})$

specific growth rate $(\mu) = \frac{\Delta X}{\Delta t} \cdot \frac{1}{X}$ (h^{-1})

$$\Delta X = X_{t_2} - X_{t_1} + D \int_{t_1}^{t_2} x\,dt \qquad (g \cdot l^{-1})$$

$$\Delta S = S_{t_1} - S_{t_2} + D \cdot S_o(t_2 - t_1) - D \int_{t_1}^{t_2} S\,dt \qquad (g \cdot l^{-1})$$

$$\Delta CO_2 = K \int_{t_1}^{t_2} P_{CO_2}\,dt \qquad (g \cdot l^{-1})$$

where

S_o = conc. of methanol in the medium feed ($g \cdot l^{-1}$)

S = conc. of methanol in the broth ($g \cdot l^{-1}$)

t = time (h)

D = dilution rate (h^{-1})

P_{CO_2} = partial pressure of CO_2 in the exhaust gas (atm)

K = conversion factor

X = cell concentration (g dry weight $\cdot l^{-1}$)

RESULTS

Dissolved oxygen. The effect of dissolved oxygen tension on the cell yield was determined under steady-state conditions. At a cell concentration around 15 $g \cdot l^{-1}$ a decrease in yield coefficient was observed when the dissolved oxygen tension reached below 0.08 atm which was a critical oxygen tension using the cell yield as a criterium. At 0.03 atm the yield coefficient was only 70 % of the maximum value obtained. Increasing the dissolved oxygen tension up to 0.5 atm had no effect on the cell yield. In these experiments the partial pressure of carbon dioxide was below 0.1 atm. In the following experiments the dissolved oxygen tension was kept above 0.13 atm.

Carbon dioxide. The cell yield from methanol was determined under steady-state conditions at different levels of carbon dioxide in the fermentation broth. A decrease in the yield coefficient was observed at a carbon dioxid partial pressure of 0.3 atm and above. At 0.6 atm the yield coefficient was only 70 % of the maximum value (0.48) observed (Fig. 1). The effect was reversible.

Methanol feed concentration. When the methanol concentration in the medium feed was increased to obtain higher steady-state cell concentrations in the chemostat a decrease in cell yield was obtained (Fig. 2). The data on yield coefficients at methanol concentrations above 80 $g \cdot l^{-1}$ are from experiments where methanol was added via a separate stream from the other medium components.

Discontinuous methanol feed. Two different ways were used for discontinuously adding methanol to the chemostat. The yield coefficient was determined during the oscillating conditions obtained. In one set of experiments the whole medium flow containing both methanol (30 $g \cdot l^{-1}$) and the other nutrients was discontinuously fed to the culture. At a pulse time (time between two additions) of 10 s a decrease in cell yield was observed (Fig. 3). When the pulse time was increased to 50 s the yield coefficient decreased to 0.39 from a maximum value of 0.48 obtained using continuous flow. In the case where methanol was fed at different intervals to the culture via a separate flow containing 280 $g \cdot l^{-1}$ methanol the yield coefficient obtained was 0.39 in the pulse time range of 0 to 80 s. This value is significantly lower than the yield coefficient 0.48 obtained when methanol was fed continuously together with the other nutrients. During the pulse experiments oscillations in dissolved oxygen tension were obtained. There were only small variations in pH and in carbon dioxide partial pressure and no measurable fluctuations in cell concentration were observed.

Transient response of a steady-state culture. Transient responses of a steady-state continuous culture was studied in more detail. A methanol pulse was added to the culture at the dilution rates 0.15 h^{-1} and 0.38 h^{-1}. During the transient period following the addition, methanol was rapidly consumed and the cell concentration and the specific carbon dioxide production increased. The calculated values of specific growth rate and yield coefficient prior to and after the pulse are shown in Fig. 4 and Fig. 5. The observed decrease in cell yield was more pronounced at the lower dilution rate. The second decrease in cell yield during the transient period occured when excess methanol had been consumed and the methanol concentration in the broth was reaching growth-limiting values. A carbon balance over the first 2.5 hours showed that carbon was missing. The culture supernatant was therefore analysed for intermediates in the energy metabolism. After the methanol pulse there was an initial accumulation of formate which was more pronounced at the lower dilution rate. The carbon recovery was close to 100 % when formate formed was included.

DISCUSSION

In response to low dissolved oxygen tensions (DOT) Methylomonas methanolica WI O showed a very sharp decrease in cell yield. A lowering of the yield coefficient was also observed when the organism was exposed to low DOT for short periods of time at regular intervals. The results are comparable to those obtained for Pseudomonas AM1 where a decrease in cell yield at low DOT was accompanied by an increase in respiration rate (Mac Lennan et al, 1971). However, the "critical" DOT reported for Pseudomonas AM1 at a cell concentration around 5 $g \cdot l^{-1}$ is lower than the value found for M. methanolica. Pseudomonas AM1 also appears to be more sensitive to high DOT tensions. Our results indicate that the measured "critical" DOT is dependent on the cell concentration used. The most probable explanation is that high cell concentrations give a larger difference in oxygen concentration between the bulk of the liquid and the cell surface than dilute cell suspensions.

Increasing the steady-state cell concentration was accompanied by a decrease in cell yield. The feeding of a concentrated methanol solution separate from the other medium components resulted in a dramatic decrease in cell yield, which shows that the organism is very sensitive to locally high methanol concentrations in the broth. Discontinuous addition at very short intervals (10 s) of the whole substrate flow including methanol decreased the biomass yield. Continuous addition of methanol with the other medium components and a good distribution of the medium feed are thus important in order to minimize gradient development in large scale fermenters.

The partial pressure of carbon dioxide had to reach relatively high levels (0.35 atm) to cause a decrease in cell yield. The effect was reversible. Critical carbon dioxide concentrations may be obtained if pure oxygen is used as the oxygen supply. Carbon dioxide has been shown to inhibit decarboxylating enzymes in vitro, probably by the way of mass action upon the enzymes involved (King and Nagel, 1975). Carbon dioxide might therefore affect the last NADH yielding step in the energy metabolism resulting in formate being excreated into the medium. This would explain why an increasing part of the methanol consumed is used for energy production at high carbon dioxide levels. In most methylotrophs the growth yield is determined partly or exclusively by NAD(P)H supply (Anthony, 1978). If the final oxidation step is the only source of NADH then the high carbon dioxide levels can also increase the probability of NADH limitation which will decrease the yield.

An analysis of the transient period following a pulse addition of methanol showed a transient accumulation of formate in the broth which show that M. methanolica WLO oxidized formaldehyde via formate and not through the cyclic oxidation that has been reported in some methylotrophs (Strøm et al, 1974). A raise in pH indicates that formic acid was oxidized.

Formate and also formaldehyde accumulation has earlier been reported for yeast growing on methanol (Held et al, 1978; Swartz, 1978). The experiments with M. methanolica WLO were performed at a low cell concentration and therefore higher formate concentration would be expected at higher cell concentrations. In batch culture where methanol is present in excess the yield coefficient for M. methanolica WLO is low which means that methanol is wasted. A raise in pH when methanol is exhausted indicates that formate is excreated during growth. According to the hypothesis put forward by Brookes and Meers P. methylotropha might be wasting energy on a constant changing of growth rate (Brookes and Meers, 1973).

The transient accumulation of formate in our experiments shows that the last NADH yielding step in the energy metabolism of M. methanolica WLO was not working optimally, which resulted in more methanol being used for energy production and decreased cell yields. As was pointed out earlier formate accumulation increases the probability of NADH limitation. This second effect of formate excretion on cell yield also lead to a waste of ATP (Anthony, 1978).

The accumulation of formate was more pronounced when a methanol pulse was administered at a low dilution rate, indicating that the enzyme involved in the last step of the energy metabolism cannot rapidly adjust its level or activity to meet the new growth rate. A pulse addition at a low steady-state growth rate also gave a more pronounced decrease in cell yield. Ebbinghaus also found that discontinuous feeding of substrate at a low steady-state growth rate resulted in a lower cell yield than when a higher dilution rate was used (Ebbinghaus, pers. comm.).

In some methylotrophs formate has been reported to inhibit methanol oxidation due to inhibition of cytochrome c oxidase (Ivanovsky et al, 1980). Inhibition of methanol oxidation could explain the slow increase in specific growth rate after the initial rapid increase following a methanol addition at the low dilution rate. This phenomena may also be due to an adjustment of enzyme levels in the cell.

The observed lowering of cell yield when organisms are exposed to methanol gradients and probably also high carbon dioxide concentrations is to a large extent caused by a non-optimally working energy metabolism. This is most likely also leading to a limited supply of necessary reducing equivalents.

Acknowledgements

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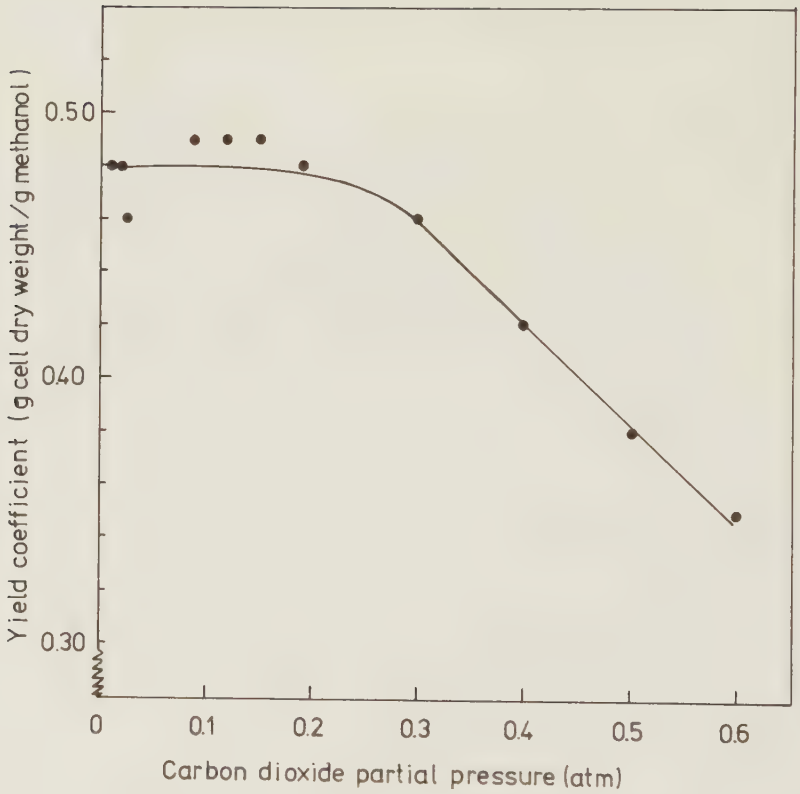

Figure 1 The yield coefficient for M. methanolica WLO in a chemostat at different carbon dioxide partial pressures. Methanol concentration in the medium feed 30 $g \cdot l^{-1}$ and DOT 0.13 atm.

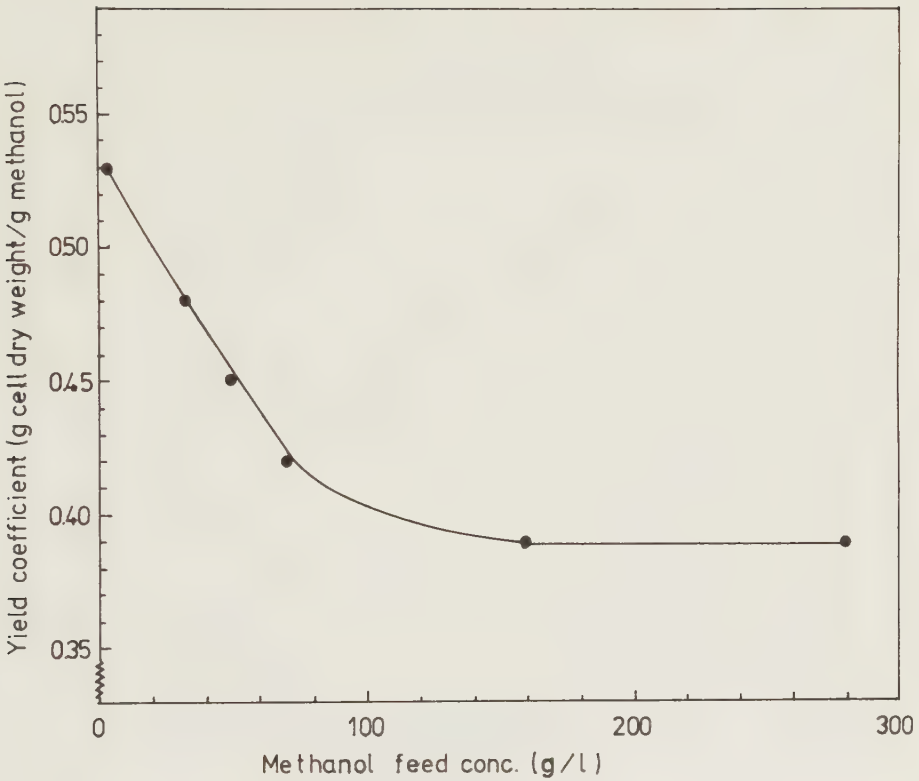

Figure 2 The yield coefficient in a chemostat culture of M.methanolica WLO at different concentrations of methanol in the medium feed.

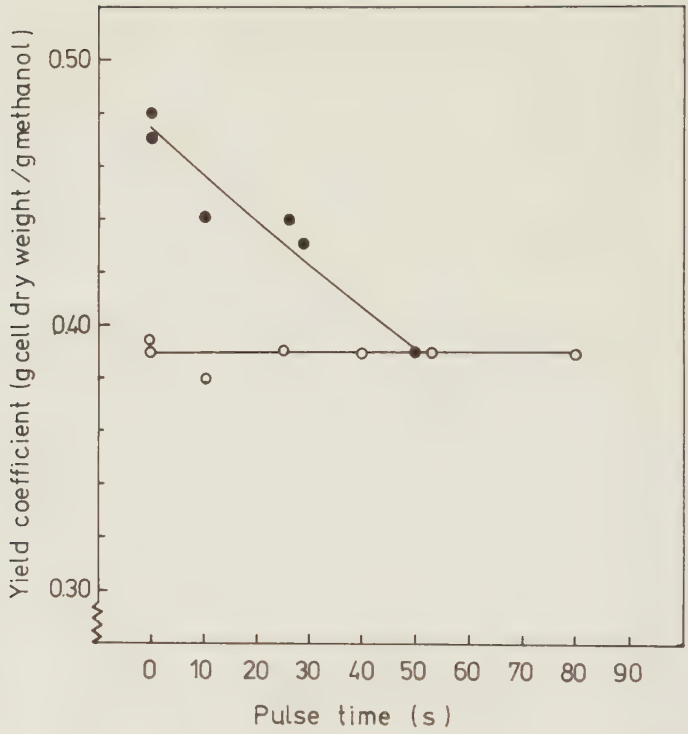

Figure 3 The apparent yield coefficient when methanol was discontinuously fed alone (closed symbols) or together with the other medium components (open symbols) to a chemostat culture of M. methanolica WLO. DOT 0.13 atm. Cell concentration 10-16 $g \cdot l^{-1}$.

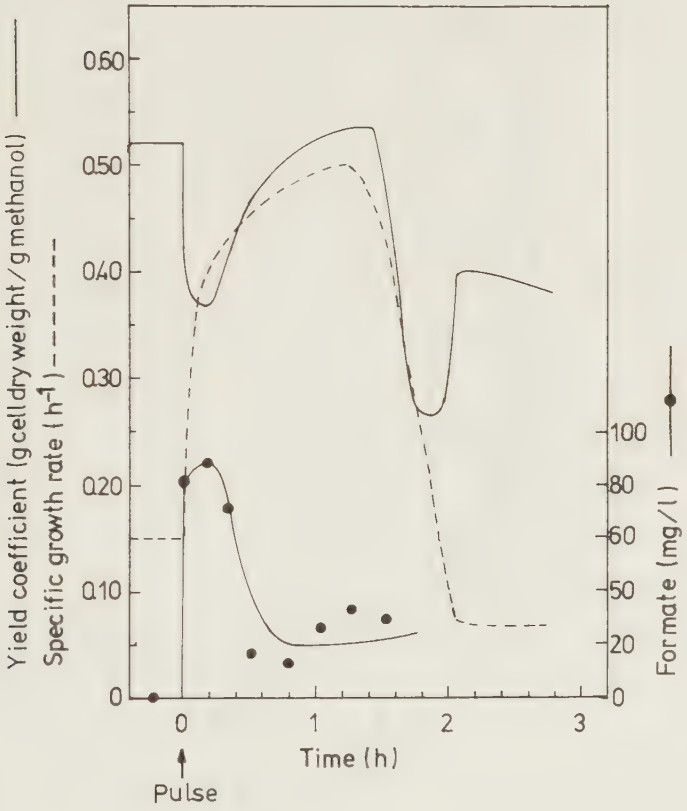

Figure 4 The transient response of M.methanolica WLO in a chemostat culture at dilution rate 0.15 h^{-1} to a pulse addition of methanol. Cell concentration 1.5 $g \cdot l^{-1}$.

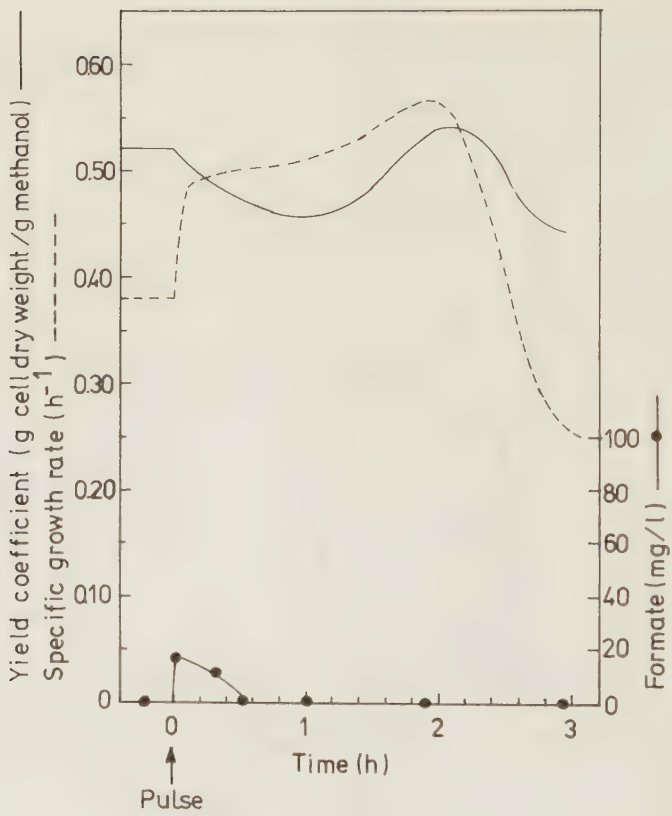

Figure 5 The transient response of M.methanolica WLO in a chemostat culture at dilution rate 0.38 h^{-1} to a pulse addition of methanol. Cell concentration 1.5 $g \cdot l^{-1}$.

II

Growth of *Streptococcus lactis* on single or mixed carbohydrates in relation to the regulation of maltose metabolism.

M.H. Häggström

SUMMARY

A pronounced formation of heterofermentative products from galactose, and especially from maltose, was found in batch cultures of S.lactis 65.1. Not only lactic acid but also ethanol, acetic acid and formic acid were formed.

Diauxic growth was occured on mixture of glucose and maltose in batch culture, whereas both sugars were consumed in maltose plus glucose-limited continuous cultures. Maltose utilization was subject to catabolite inhibition mediated by glucose. An intracellular inducible maltose phosphorylase was found. The maltose molecule is probably transported by a permease into the cell where it is split. Maltose phosphorylase was not induced in the presence of glucose in the medium and the results presented lend support to the theory that the synthesis is under the control of catabolite repression.

Transient responses in continuous culture showed that the mixed substrate system was sensitive to shift-ups in dilution rate which caused incomplete utilization of the carbohydrate mixture, and may be due to the catabolite repression and inhibition controls involved.

INTRODUCTION

The group N-streptococci *Streptococcus lactis*, *S.cremoris* and *S.diacetylactis* are important organisms in the dairy industry where they produce L-lactic acid from lactose in milk.

In these organisms the metabolism proceeds from hexose through the Emden-Meyerhof-Parnas pathway. Carbohydrates are phosphorylated either by the phosphoenolpyruvate dependent phosphotransferase system during transport or by ATP after being transported into the cell (Lee et al. 1973; Thompson et al. 1978).

N-streptococci are regarded as homolactic fermenters and in batch culture around 90 % of lactose or glucose consumed is fermented to L-lactic acid. Heterolactic fermentation occurred when *S.lactis* was metabolizing galactose or when *S.lactis* 7962, which lacks a phospho-β-galactosidase, was growing on lactose (Thomas 1976). Chemostat studies where glucose was the growth limiting substrate showed an increase in the heterolactic fermentation when the dilution rate was decreased. Depending on the strain used this shift was more or less pronounced (Thomas et al. 1979).

Utilization of mixed carbohydrates in batch cultures of S.lactis ML_3 has been reported. Growth on a mixture of glucose, lactose and galactose has shown a sequential utilization of the sugars (Thompson et al. 1978).

The present study involves carbohydrate utilization and product formation by S.lactis 65.1 in batch cultures on single and mixed carbohydrates. No reports are available on the growth of and product formation by S.lactis on maltose and mixtures of maltose and glucose, which are the fermentable sugars in hydrolyzed starch. It was therefore decided to study the regulation of maltose utilization and the maltose-splitting enzyme involved.

MATERIALS AND METHODS

Organism

The organism used was Streptococcus lactis 65.1, which was obtained from the Swedish Dairies Association, Malmö, Sweden. S.lactis was stored in lithmus milk at -20° C.

Growth media

The medium used for preparing inoculum and for fermenter cultivations contained in $g \cdot l^{-1}$: tryptone 5.0, yeast extract 5.0, casamino acid 1.0 (all products of Difco Laboratories), K_2HPO_4 2.5, KH_2PO_4 2.5, $MgSO_4 \cdot 7H_2O$ 0.5 and carbohydrate around 10.0 is indicated in the text. This medium supported growth at a sugar concentration of 20 $g \cdot l^{-1}$. In the continuous culture experiments the medium was sterilized for 40 minutes.

Growth conditions

The batch and continuous cultures were performed in fermenters (Chemoferm AB, Hägersten, Sweden) with working volumes of 2.5 and 0.7 litres. The pH was kept constant at 6.5 by addition of sodium hydroxide and the temperature was 30° C. Anaerobic conditions were maintained by passing nitrogen through the head-space of the fermenter. In the continuous culture (chemostat) experiments the volume was controlled using a conductivity type level controller.

Growth measurements

Culture turbidity was measured in a Turner photometer

at 620 nm. To determine the cell dry weight a defined volume of cell suspension was centrifuged, the cells were washed, dried at 105° C for 12 h and weighed.

Analyses of carbohydrates and metabolic products

When taking samples from the fermenter the cells were quickly removed by centrifugation followed by membrane filtration. The supernatant was analysed for substrates and products. Glucose was measured with the use of a hexokinas/glucose 6-phosphate kit. The L-lactate-hydrogenase based kit was used to measure L-lactic acid (both products of Boehringer Mannheim, Germany). Maltose was assayed as glucose following a hydrolytic step using α-glucosidase (Sigma Chemical Co., Mo, USA). Galactose was assayed with galactose dehydrogenase according to the method described by Kurz and Wallenfels (1974). Formic acid was determined according to Lang and Lang (1972). Acetic acid and ethanol were assayed with a Varian 940 gas chromatograph equipped with a 1/16" i.d. 8 feet long teflon column containing Chromosorb 101. The column temperature was 160° C and nitrogen saturated with formic acid was used as carrier gas at a flow rate of 30 $ml \cdot min^{-1}$.

Preparation of cell-free extracts

Thirty millilitres of cell suspension was removed from the growing culture, centrifuged for 10 min at 15,000 g, 4° C and washed with 0.04 M sodiumphosphate buffer pH 6.8. The pellet was suspended in 3 ml of the same buffer, 2.5 ml glass beads (0.2 mm) were added, and the cells were disintegrated for 3 minutes on ice in an ultrasonic disintegrator (MSE, Crawley, England). Cell debris was removed by centrifugation at 30,000 g, 4° C for 30 min. The maltose hydrolysing activity was determined in the supernatant. The protein concentration in the cell-free extract was measured according to Lowry et al. (1951) using bovin serum albumin fraction V (Boehringer, Mannheim) as the standard.

Assay of maltose hydrolysing activity

Hydrolysing activity was determined in cell-free extracts. The following standard assay mixture was used in 1 ml: 0.1 M sodium phosphate buffer pH 6.5, 0.2 M maltose and cell-free extract. The reaction was run at 30° C and was initiated by addition of maltose and stopped by heating to 90° C. Glucose standard and substrate blank were treated in the same way. Glucose released was assayed using the hexokinase/glucose 6-phosphate kit (Boehringer, Mannheim). One mole of

maltose was assumed to give 1 mole of glucose, and one unit of enzyme activity was defined as the amount of enzyme that splits 1 µmole maltose per minute. Assays were carried out on duplicate cell-free extracts, and the activity was proportional to the enzyme concentration. The assayed *in vitro* activity was used as a measure of the enzyme level in the cell.

RESULTS

Batch culture on single carbohydrate

Growth and product formation of *S. lactis* 65.1 on various sugars was studied. *S. lactis* 65.1 fermented glucose, lactose, galactose and maltose but did not metabolize sucrose or mannitol. Growth on glucose and lactose showed a homofermentative fermentation pattern, whereas product formation from galactose and maltose was heterofermentative (Table 1). The main products formed from maltose and galactose were L-lactic acid, acetic acid, ethanol and formic acid. Maltose gave the most pronounced heterofermentative product pattern. The measured maximum specific growth rates on the different sugars were: 9.75 h^{-1} for glucose and lactose, 0.62 h^{-1} for galactose and 0.47 h^{-1} for maltose.

The data on product formation from maltose and galactose agreed with a metabolic route, where pyruvate undergoes the clastic reaction to formate and acetyl-CoA (Fig. 1). The energy balance on NAD/NADH was 95 and 103 % for maltose and galactose, respectively. Around 95 % of the substrate consumed was metabolized to products. Growth on maltose and galactose also showed a higher growth yield compared with growth on glucose. Assuming a constant ATP yield the higher growth yield may be explained by the additional ATP formation when acetate is formed.

Batch culture on mixed carbohydrates

When S. lactis 65.1 was grown on a mixture of glucose, lactose and galactose the sugars were utilized sequentially. Glucose and lactose were consumed simultaneously whereas galactose utilization started when glucose, but not lactose in the medium had been exhausted. Diauxic growth occured when the organism was supplied with a mixture of glucose and maltose (Fig. 2) and not before 1.5 hours later did maltose utilization start. Maltose metabolism was accompanied by a switch from lactate to acetate, ethanol and formate formation. In Figure 2 heterofermentative product formation is represented by acetate.

Maltose utilization

When the organism had been growing on glucose induction was most probably required before maltose utilization could start (Fig. 2). An experiment was carried out where protein synthesis was stopped by adding chloramphenicol to a culture growing on glucose in the presence of maltose. When glucose had been consumed no maltose utilization occurred showing that the enzymes involved in maltose metabolism are inducible.

The maltose-splitting enzyme of S.lactis 65.1 was found in cell-free extracts after sonication. Cells growing in batch culture on glucose alone or on glucose in the presence of maltose showed no maltose hydrolyzing activity.

Addition of a pulse of glucose to a culture growing on maltose immediately completely inhibited the utilization of maltose (Fig. 2). However, in the in vitro system the maltose splitting enzyme was not inhibited by glucose in the concentrations lower than 1 $g \cdot l^{-1}$, the concentration used in the pulse experiment.

Steady-state continuous culture

Growth and substrate utilization were studied in steady-state chemostat cultures. The growth limiting substrates were glucose, maltose and a mixture of glucose and maltose in approximately equal amounts.

In the glucose-limited culture residual glucose was around 60 mg/l with a small increase when the dilution rate reached 0.7 h^{-1}.

When maltose was the growth limiting component the residual maltose concentration increased much more with dilution rate. There was a linear increase in specific maltose utilization rate up to about 0.5 h^{-1} (Fig. 3). The interrupted lines in the figure indicate that the culture was approaching unsteady-state conditions.

In a chemostat culture supplied with a mixture of glucose and maltose both sugars were consumed simultaneously, showing that the enzymes necessary for maltose metabolism were synthesized in the presence of low concentrations of glucose (below 60 $mg \cdot l^{-1}$) (Fig. 4). The specific maltose utilization rate increased up to 0.6 h^{-1} and the increase in residual maltose concentration started at a somewhat higher dilution rate than in the maltose-limited culture.

Transient response

Transient conditions were induced in steady-state chemostat cultures by performing shift-ups in dilution rate (D). The shift was around 0.15 h^{-1}. In a maltose-limited culture the dilution rate was shifted from 0.05 to 0.21 h^{-1} and from 0.37 to 0.50 h^{-1} (Fig. 5). The former increase was followed by a peak increase whereas the latter shift resulted in a gradual increase in the concentration of residual maltose. The calculated increase in specific maltose consumption rate was slow at the low and rapid at the high dilution rate shifts. These results indicate that enzyme synthesis was necessary for increasing the growth rate from 0.05 to 0.21 h^{-1} but not for the higher shift.

Shift-up experiments from 0.1 to 0.25 h^{-1} were also performed in a chemostat culture limited by a mixture of glucose (4.8 $g \cdot l^{-1}$) and maltose (4.1 $g \cdot l^{-1}$) as well as in a glucose-limited culture (Fig. 6 A and B). In the mixed substrate culture there was no maltose consumption during the first two hours after the shift. In contrast to the response of the glucose-limited culture (Fig. 6 B), no increase occurred in glucose concentration (Fig. 6 A) showing that before the shift the enzymes necessary for

glucose metabolism were present at levels corresponding to the total flow of carbohydrate in the cell. Performing the shift-up experiments from 0.25 to 0.41 h^{-1} resulted in a cessation of maltose utilization and a gradual increase in residual maltose concentration, and there was no accumulation of glucose. In the glucose-limited culture a similar increase in the dilution rate was followed by a small transient increase in the glucose concentration. The results indicate that an increased glucose uptake inhibited the maltose metabolism and that the catabolism of maltose and glucose mainly involves the same enzymes, except for those specific for maltose metabolism.

Enzyme activity

As the enzyme metabolizing maltose was found to be inducible its activity was followed under different growth conditions. The hydrolytic enzyme was found to be dependent on inorganic phosphate for activity indicating that it is a phosphorylase.

The maltose phosphorylase activity in a continuous maltose-limited chemostat and in a double substrate-limited chemostat are shown in Fig. 3 and Fig. 4

respectively. When the dilution rate was increased the cells increased their specific maltose consumption rate by increasing the maltose phosphorylase level of the cell. When the maltose phosphorylase level had reached a maximum value the specific maltose uptake rate increased with the residual maltose concentration.

Assuming a 70 % protein content of the cell the *in vivo* reaction rates can be compared with the maximum calculated rates in *in vitro* studies. Maximum *in vivo* maltose consumption rates were only 39 % of the calculated values. At the low residual maltose concentrations the *in vivo* activity was only around 11 % of the activity measured *in vitro*. These results apply to maltose-limited as well as maltose plus glucose-limited chemostats.

The maltose phosphorylase activity in cells growing at maximum rate in a batch culture on maltose was $43 \cdot 10^{-2}$ units/mg protein compared with $52 \cdot 10^{-2}$ units/mg protein, which was the maximum value noted in a maltose-limited culture.

Maltose phosphorylase activities in a maltose-limited culture were compared with the activities measured in

cells growing on maltose and glucose. At the same residual maltose concentration the enzyme activity was proportional to the flow of maltose through the cell The maximum specific maltose consumption was also proportional to the enzyme level in the two different chemostat cultures. The results indicate that the maltose metabolism was limited by the maltose phosphorylase activity of the cell.

DISCUSSION

In batch culture the fermentation of glucose by S. lactis 65.1 was homofermentative. Growth on maltose, however, caused a dramatic shift towards a heterofermentative fermentation pattern. In addition to lactate, substantial amounts of acetate, ethanol and formate were formed. Galactose growth gave a lactate yield which was between the yields obtained on glucose and maltose. The metabolic route to acetate with the liberation of formate is known to be present in S. mutans (Yamada and Carlsson 1975) and has recently been reported in S.lactis (Thomas et al. 1979).

Diauxic growth was obtained in batch culture when S. lactis 65.1 was supplied with a mixture of glucose and

maltose or a mixture of glucose, galactose and lactose. Maltose and galactose were not metabolized in the presence of glucose and when utilization started there was an immediate relative increase in acetate, ethanol and formate formation. Induction of one or more enzymes specific for the maltose metabolism was necessary for maltose utilization to start. In contrast, in continuous culture limited by a mixture of glucose and maltose both sugars were consumed simultaneously.

In S. lactis 65.1 maltose was shown to induce a maltose phosphorylase, which was not synthesized when glucose was present on the broth. Maltose consumption was immediately inhibited on addition of glucose to the culture showing that the maltose metabolism was subjected to catabolite inhibition (McGinnis and Paigen 1969). The maltose phosphorylase activity in vitro was not inhibited by its reaction products in the concentration range tested, < 1.0 g/l.

Several mechanisms for regulating carbohydrate uptake in bacteria have been described. The enzyme III^{glu} of the phosphoenolpyruvate (PEP) dependent glucose phosphotransferase system (PTS) was shown to be a regulatory protein inhibiting permeases in Escherichia coli. Cyclic

adenosine 3´,5´-monophosphate (cAMP) promoted desensitization of transport systems subjected to PTS-mediated regulation (Dills et al. 1980). S.lactis 65.1 showed PTS activity for glucose (Häggström, unpublished data). The use of PTS for transport of glucose and lactose has been reported for S.lactis ML_3. Galactose may be transported by a permease or via the lactose PTS (Thompson 1978). Cell-free extract of S.lactis 65.1 proved to hydrolyze maltose and assuming the enzyme to be specific for maltose it appears likely that maltose is transported by a permease into the cell, where it is split.

The absence of maltose phosphorylase synthesis in S.lactis 65.1 in the presence of glucose is most probably due to either inducer exclusion caused by PTS-mediated regulation of maltose uptake or catabolite repression mediated by glucose (Mazasanik 1976). The regulation may involve cAMP. Cyclic-AMP activity has been observed in S.lactis C2 and other N-streptococci and elevated intracellular cAMP levels prevented repression of enzyme synthesis by glucose (Timothy et al. 1980).

A shift-up in dilution rate in a glucose plus maltose-limited chemostat was accompanied by an immediate inhibition of maltose consumption but there was no

accumulation of glucose. It was an increase in glucose uptake rate, and not an increase in glucose concentration that caused the inhibition of maltose utilization. These results lend support to the theory that glucose transport inhibits maltose uptake.

The transient responses noted following shift-ups in dilution rate in maltose- and maltose plus glucose-limited chemostats indicated that an increase in growth rate from a low level requires enzyme synthesis. Cells growing at a higher rate increased their growth rate by increasing the residual maltose concentration. In chemostats limited by maltose or maltose plus glucose the maltose phosphorylase level likewise varied with the dilution rate. Initially there was an increase in activity up to a maximum value, which was followed by a slight decrease at higher dilution rates. These results agree with the observed transient behaviour following shift-ups in dilution rate under different conditions.

Hypothetically, the induction of a rate-limiting enzyme should increase with the dilution rate. However catabolite repression (Mazasanik 1976) would presumably increase with the dilution rate. The maltose molecule is split intracellularily into glucose and probably glucose-1P, where

glucose or a subsequent metabolite is known to mediate catabolite repression. Results from maltose-limited chemostats showed that synthesis of maltose phosphorylase was most likely under catabolite repression control, which was probably mediated by the glucose moiety released intracellularily when maltose was cleaved. Inducer exclusion can be ruled out when the culture is growing on maltose. The findings in a batch culture, where the enzyme activity was lower than the maximum value obtained in continuous culture, also argue for catabolite repression of the maltose phosphorylase synthesis. Also data from chemostats on the mixed substrate support the catabolite repression theory.

The maximum maltose phosphorylase activity in the two different chemostats was proportional to the flow of maltose through the cell. There was a difference between the maximum *in vitro* maltose splitting rate and the measured *in vivo* maltose consumption rate, which showed that maltose phosphorylase in the cell was not working at maximum rate. The intracellular maltose concentration was most likely rate limiting, which is also supported by the fact that the difference between the *in vitro* and *in vivo* activities decreased with increasing residual maltose concentration at a constant maltose phosphorylase level.

The present study shows that the choice of raw material and growth conditions are important for product formation by bacteria in fermentation processes. When using S. lactis 65.1 as starter culture on substrates containing hydrolyzed starch the yield of the different products will depend on the degree of hydrolysis.

The transient experiments in continuous culture on mixed carbohydrates show that the carbohydrate utilization was sensitive to changes in growth rate which especially applies to mixed carbohydrate systems where one of the sugars mediates catabolite repression and/or inhibition. Thus gradient formation caused by e.g. insufficient mixing, irregular addition or feeding of a concentrated substrate solution in large scale fermentations may result in incomplete utilization of one or more carbohydrates in the mixture.

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Table 1. Yields of products in batch cultures on different sugars. The figures denote percentage of theoretical values.

Sugar	L-lactate	Acetate	Ethanol	Formate
Glucose	94	< 1	< 1	< 1
Lactose	92	< 1	< 1	< 1
Galactose	63	13	16	27
Maltose	39	35	29	63

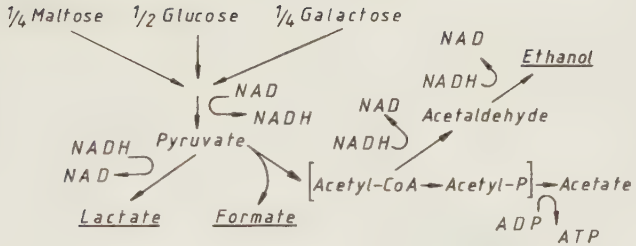

Figure 1 Suggested heterofermentative metabolic pathway in S.lactis 65.1.

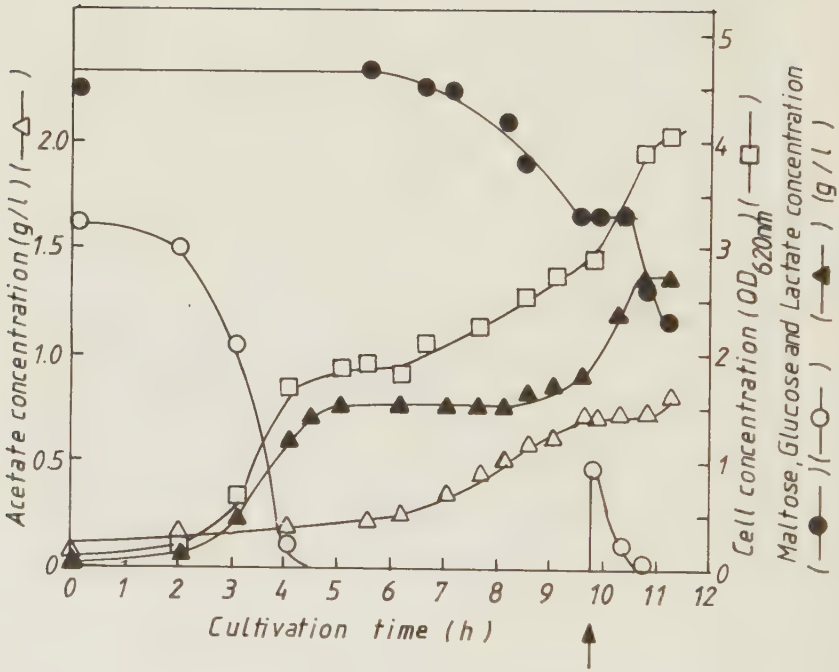

Figure 2 Batch culure of S. lactis 65.1 on a mixture of glucose and maltose. The arrow indicates a pulse addition of glucose.

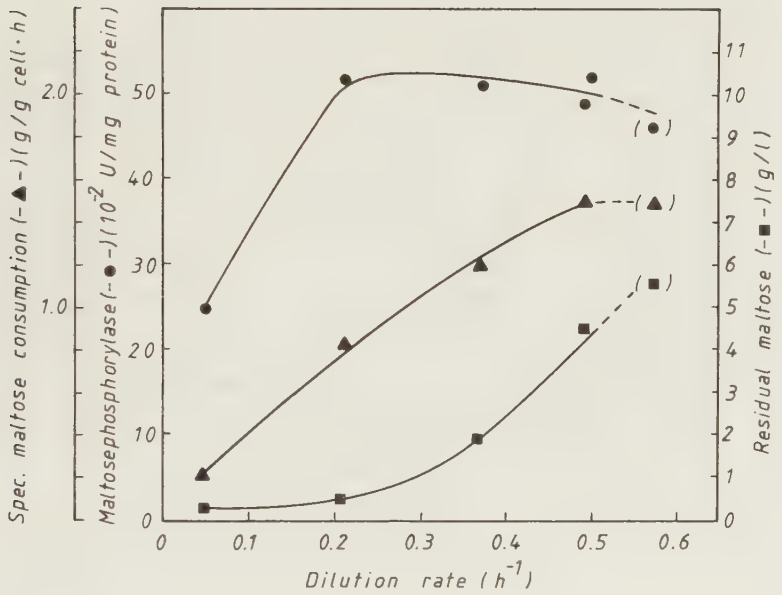

Figure 3 Maltose phosphorylase activity, residual maltose concentration and specific consumption rate in a maltose-limited chemostat of S. lactis 65.1. Maltose concentration in substrate feed 8.0 $g \cdot l^{-1}$.

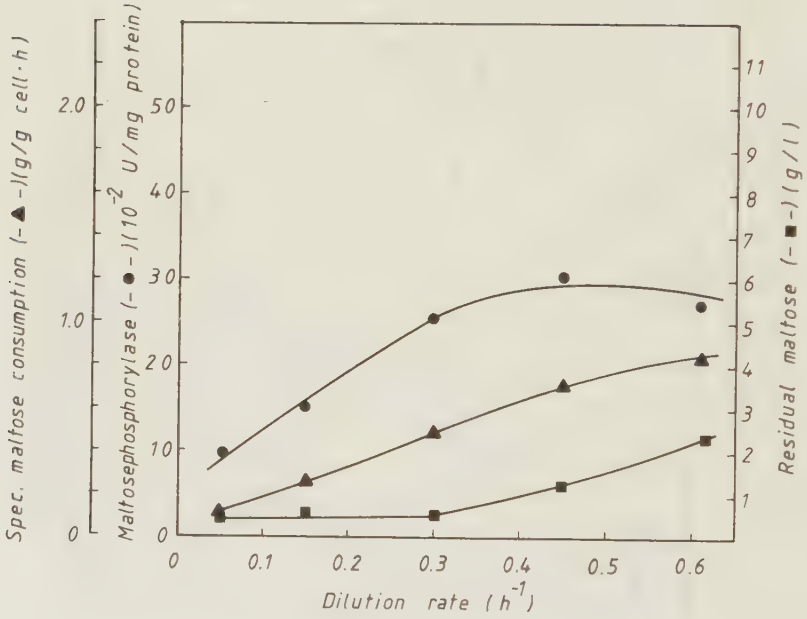

Figure 4 Maltose phosphorylase activity, residual maltose concentration and specific maltose consumption rate in a glucose plus maltose-limited chemostat of S. lactis 65.1. Concentration in the substrate feed of glucose 5.0 g·l^{-1} and maltose 4.1 g·l^{-1}. Residual glucose concentration <60 mg·l^{-1}.

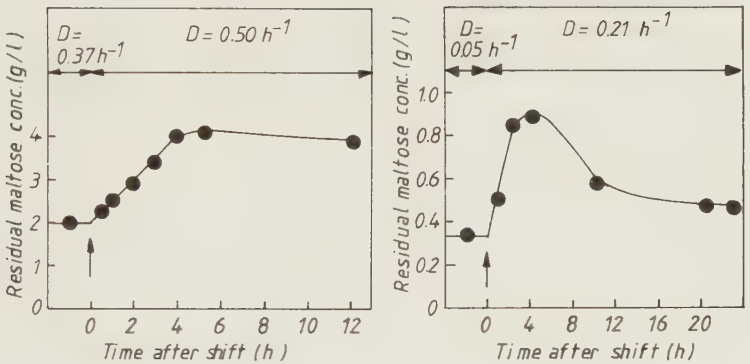

Figure 5 Transient responses of S. lactis 65.1 in a maltose-limited chemostat after shift-ups in dilution rate from 0.05 to 0.21 h^{-1} (A) and 0.37 to 0.50 h^{-1} (B). Maltose in substrate feed 7.1 $g \cdot l^{-1}$.

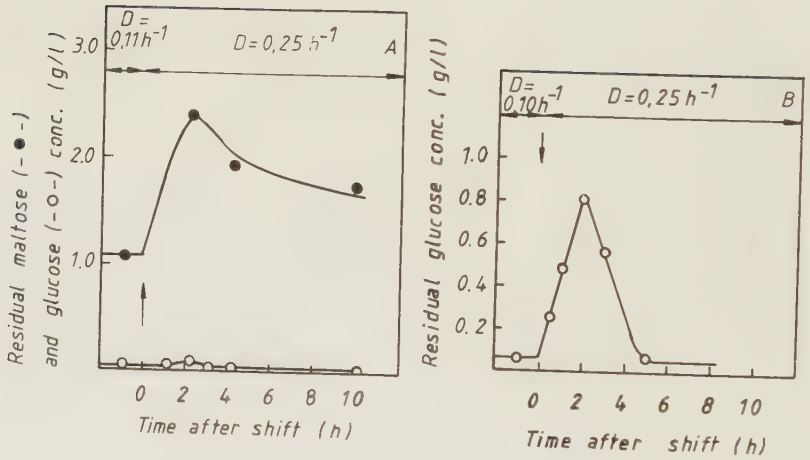

Figure 6 Transient responses of chemostat cultures of S. lactis 65.1 limited by maltose (4.1 $g \cdot l^{-1}$) + glucose (4.8 $g \cdot l^{-1}$) (A) and glucose (8.4 $g \cdot l^{-1}$) (B) after a similar shift-up in dilution rate.

III

Heterofermentative product formation in *Streptococcus lactis* and the regulation of glycolysis in maltose metabolism.

M.H. Häggström

ABSTRACT

Streptococcus lactis 65.1 showed a constant and pronounced heterofermentative product formation (lactate yield 20 to 25 %) in the dilution rate range of 0.05 - 0.50 h^{-1} when growing in a maltose-limited chemostat. In corresponding glucose-limited culture the lactate yield increased from 65 to 85 % with the dilution rate.

The L-lactate dehydrogenase (L-LDH) from S.lactis 65.1 was activated by fructose 1,6-diphosphate (FDP) and inhibited by orthophosphate. Pyrophosphate was also found to be an inhibitor. An inducible maltose phosphorylase requiring around 50 mM orthophosphate for maximum activity was present in maltose growing cells.

Orthophosphate was shown to affect product formation in vivo. Phosphate involved in the intracellular splitting of the maltose molecule was proposed to play the main role in regulating the glycolysis away from lactic acid formation in cells growing on maltose. In addition to affecting L-LDH activity phosphate probably inhibited the pyruvate kinase which may alter the intracellular pyruvate pool.

INTRODUCTION

N-streptococci (Streptococcus lactis, S.cremoris and S. diacetylactis) are regarded as homofermentative lactic acid

bacteria and are used in starter cultures for the dairy industry.

Heterofermentative product formation in Streptococcus lactis has been reported in glucose-limited continuous culture (13) and in batch culture on galactose (12). In a previous study heterofermentation was observed in batch culture of S.lactis 65.1 growing on galactose or maltose (M. Häggström, unpublished data). Around 35 % of the maltose consumed was converted to lactic acid. The other products formed were ethanol, acetic acid and formic acid.

L-lactate dehydrogenase (L-LDH) and pyruvate kinase, which are involved in the reduction and formation of pyruvate, respectively, are regulatory enzymes in the glycolytic pathway of N-streptococci. In vitro characterization of L-LDH from S.cremoris (7) and S.lactis (4) showed that the glycolytic intermediate fructose 1,6-diphosphate (FDP) activated the enzyme. Orthophosphate inhibited the FDP activated LDH. The optimal range of pH for L-LDH from these organisms were broad in the presence of FDP. In glucose-limited chemostat culture S.lactis reportedly changes intracellular FDP pool and LDH level with the dilution rate (12). Pyruvate kinase from S.lactis (2, 3) and S.cremoris (12) is activated by FDP and other sugarphosphates and the activated pyruvate kinase is strongly inhibited by phosphate.

This paper concerns product formation in continuous culture

by S.lactis 65.1 growing on glucose, maltose or a mixture of the two sugars and discusses the heterofermentative product formation obtained in relation to the regulation of the glycolytic pathway in N-streptococci.

MATERIALS AND METHODS

Organism

The organism used was Streptococcus lactis 65.1 from the Swedish Dairies Association, Malmö, Sweden. S.lactis was stored in lithmus milk at -20^0 C.

Medium

The medium used for growing inocula and for fermenter cultivation contained ($g \cdot l^{-1}$): Tryptone 5.1, yeast extract 5.1, casamino acid 1.0 (all products of Difco Laboratories) K_2HPO_4 2.5, KH_2PO_4 2.5, $MgSO_4$ 0.24 and carbohydrate, as indicated in the text. For the continuous cultivations the medium was sterilized for 40 minutes.

Cultivation conditions and measurements

The batch and continuous chemostat cultivations were performed in fermenters (Chemofern, Hägersten, Sweden) with working volumes of 2.5 and 0.7 litres. Anaerobic conditions were maintained by passing nitrogen and air, respectively,

through the head-space of the fermenter. The temperature was 30° C and the pH was kept constant at 6.5 with NaOH. A conductivity type of level controller was used for keeping the volume in the fermenter constant. The growth was followed by turbidity measurements at 620 nm in a Turner photometer and by weighing the cells after they had been washed and dried at 105° C for 12 h.

Preparation of cell-free extracts

Thirty millilitres of cell suspension was removed from the growing culture, centrifuged for 10 min at 15,000 g, 4° C and washed with 0.04 M sodium phosphate buffer, pH 6.8. The pellet was suspended in 3 ml of the same buffer, 2.5 ml glass beads (0.2 mm) was added, and the cells were disintegrated for 3 minutes on ice in an ultrasonic disintegrator (MSE, Crawley, England). Cell debris was removed by centrifugation at 30,000 g, 4° C for 30 min. In the cell-free extract obtained as supernatant the protein concentration was measured according to Lowry et al. (10) with bovine serum albumin fraction V (Boehringer, Mannheim, Germany) as standard.

Enzyme activities in cell-free extracts

Lactate dehydrogenase (LDH) and maltose phosphorylase activities were determined immediately after the preparation of cell-free extracts. Assays were carried out on duplicate

samples and the activity was proportional to the enzyme concentration.

The standard LDH assay system contained 67 mM Tris (hydroxymethyl)-aminomethane hydrochloride pH 7.0, 0.16 mM NADH, 0.33 mM FDP tetracyclohexyl ammonium salt, 33 mM sodium pyruvate and cell-free extract in 3 ml. The reaction was initiated by adding cell-free extract and was run at 25° C. NADH oxidation was followed at 340 nm and was corrected for NADH oxidase activity (aerobically grown cells) found when pyruvate was omitted from the assay system. One unit of LDH activity was defined as the amount of enzyme that oxidizes 1 μmole NADH/min.

For maltose phosphorylase the following assay system was used in 1 ml: 0.1 M sodiumphosphate buffer pH 6.5, 0.2 M maltose and cell-free extract. The reaction was run at 30° C and was initiated by adding maltose and stopped by heating the system to 90° C. Glucose standard and substrate blank were treated in the same way. One mole of maltose was assumed to give 1 mole of glucose and glucose released was assayed. One unit of maltose phosphorylase activity was defined as the amount of enzyme that splits 1 μmole maltose/min.

The assayed LDH and maltose phosphorylase activities were used as measures of the enzyme levels in the cell.

Assays of glycolytic intermediates

Fructose 1,6-diphosphate (FDP) and glucose-1-phosphate were measured. The procedure described by Yamada and Carlsson (17) was used for determining FDP in the cells. A cell suspension was removed from the fermenter and centrifuged at 15,000 g, 4° C for 10 min. After removal of the supernatant the cells were immediately extracted with perchloride acid. When calculating the intracellular FDP concentration we used the following relationship given by Thompson (14) for S.lactis: 1 g cell dry weight was equivalent to 1.67 ml intracellular fluid. The α-anomer of glucose-1-phosphate was assayed according to Bergmeyer and Michal (1) with the addition of 1 µM of the coenzyme glucose 1,6-diphosphate.

Thin-layer chromatography

Glucose, maltose and glucose-1-phosphate, which are the components involved in the maltose phosphorylase reaction, were determined qualitatively on HPTLC Kiselgel 60 plates (Merek, Darmstadt, Germany). The solvent system was butanol: acetic acid: water (2:1:1) and the plate was developed by spraying it with a solution of 5 % sulphuric acid in ethanol and heating at 105° C.

Analyses of carbohydrates and metabolic products

Cells were rapidly removed from the fermenter samples by

centrifugation and membrane filtration, and the supernatants were assayed for substrate(s) and products. Glucose and L-lactate were determined with kits based on hexokinase/glucose 6-phosphate and L-lactate dehydrogenase, respectively (Boehringer, Mannheim, Germany). Maltose was assayed as glucose following a hydrolysis by α-glucosidase (Sigma Chemical Co, USA). Formic acid was assayed according to Lang and Lang (8). Acetic acid and ethanol were determined on a Varian 940 gas chromatograph equipped with a 1/16 inch I.D. 8 feet long teflon column containing Chromosorb 101. The column temperature was 160° C and nitrogen saturated with formic acid was used as carrier gas at a flow rate of 30 $ml \cdot min^{-1}$.

RESULTS

Product formation in continuous culture

S.lactis 65.1 was grown in steady-state anaerobic chemostat cultures in which glucose, maltose or a mixture of maltose and glucose were growth-limiting. The total sugar concentration was 10 $g \cdot l^{-1}$ and in the mixed substrate 45 % was maltose and 55 % glucose (Fig. 1). The degree of heterofermentation is represented by acetate plus ethanol relative to the theoretical yield. Lactate and formate were the other two main products. The yields of acetate plus ethanol in the maltose-limited culture did not vary appreciably with the dilution rate and thereby differed from the results obtained

in the other chemostats. There was a high degree of heterofermentation. When the organism was supplied with a mixture of maltose and glucose the variation of the acetate plus ethanol yield with the dilution rate appeared to be similar to that found in the glucose-limited culture. The acetate and ethanol yields obtained in the mixed substrate system were between those noted on the single sugars. The carbon balances in the different chemostats using substrate consumed and products formed showed a 90 to 100 per cent recovery.

The results from the aerobic glucose-limited continuous culture (Table 1) differed from those obtained in the corresponding anaerobic culture in that no ethanol and only small amounts of formic acid were detected when acetate was formed. A balance on sugar and product carbon showed that at lower dilution rates unidentified products must be formed. The balance on NAD-NADH was not closing which can be explained by the presence of a NADH-oxidase in aerobically grown S.lactis 65.1.

Results obtained in the continuous cultures limited by maltose or maltose plus glucose showed a 1:1 product ratio of formate to acetate plus ethanol, which is in agreement with a proposed main metabolic route from pyruvate to acetate via a phosphoroclastic reaction. Similar results were obtained in batch cultures of S.lactis 65.1 on galactose and maltose (M. Häggström, unpublished data). This ratio was around 0.7 in the anaerobic and around 0.4 in the aerobic

glucose-limited chemostats. Carbon dioxide was shown to accumulate in a glucose-limited chemostat where no gas was passed through the head-space. In addition, a carbon dioxide, instead of a nitrogen atmosphere, resulted in an increased lactate yield in a glucose-limited culture. These results show that acetate can be formed from pyruvate via decarboxylation.

L-lactate dehydrogenase in vitro

Studies using cell-free extract were carried out in vitro to characterize the L-lactate dehydrogenase from S.lactis 65.1. Addition of the glycolytic intermediate fructose 1,6-diphosphate (FDP) to the assay system resulted in an increased L-LDH activity (Fig. 2). The effect of inorganic phosphate on the L-LDH activity in the presence of different concentrations of FDP are summarized in Fig. 3, where the values at each FDP concentration were related to the maximum LDH activity at that FDP concentration. At low FDP levels there was a striking decrease in LDH activity already at a low phosphate concentration. A similar response occurred when pyrophosphate was added to the assay system at different FDP concentrations. The activation of L-LDH by FDP was inhibited by inorganic phosphate and pyrophosphate. The former was the stronger inhibitor.

When the intracellular FDP concentration had been determined in cells from batch cultures of S.lactis 65.1 the effect of

pyruvate concentration on L-LDH activity was measured. At the FDP concentrations 5 and 15 mM and NADH concentration 0.16 mM the K_m value for pyruvate was around 1.2 mM. Addition of 67 mM orthophosphate in the presence of 5 mM FDP and 0.16 mM NADH doubled the K_m value for pyruvate.

Maltose phosphorylase

A balance on maltose consumed and glucose formed by a cell-free extract of S.lactis 65.1 showed a molar ratio of 1:1. The reaction was phosphate dependent, indicating that the enzyme involved is a phosphorylase (Fig. 4). Thus the other reaction product must be glucose-1-phosphate (glu-1P), which was confirmed qualitatively by thin-layer chromatograph.

An attempt to measure glu-1P enzymatically failed. As phosphoglucosemutase is specific for the α-anomer of glu-1P, the product formed by the maltose phosphorylase might have been β-glucose 1-phosphate (β-glu-1P).

The following reverse reaction experiment was therefore carried out at 30° C in Tris (hydroxymethyl) aminomethane buffer pH 7.0 with the use of cell-free extract of S.lactis 65.1 grown on maltose:

α-glu-1P (11 mM) + glucose (100 mM) → maltose + phosphate

β-glu-1P (11 mM) + glucose (100 mM) → maltose + phosphate

Maltose, glucose and glu-1P were determined qualitatively

by thin layer chromatography. Maltose was formed only from β-glu-1P and glucose. Thus, the products of the maltose phosphorylase reaction in S.lactis 65.1 were glucose and β-glucose-1-phosphate.

L-LDH levels and FDP pools in vivo

Being one of the regulatory enzymes in the glycolytic pathway in S.lactis, L-lactate dehydrogenase (L-LDH) was measured under different growth conditions. Fructose 1,6-diphosphate is an activator of the L-LDH in vitro and was, therefore measured in cells growing on sugar in excess.

In batch culture the same FDP levels were found in galactose-, glucose- and maltose growing cells (Table 2). In cells growing exponentially on galactose or maltose the LDH levels were the same (Table 2). Similar LDH levels were also found in cells harvested at the same dilution rate from the different anaerobic chemostats in the dilution rate range of 0.05 - 0.50 h^{-1} (Fig. 5). Compared with the corresponding anaerobic glucose-limited culture the corresponding aerobic culture (Table 1) showed similar LDH levels at the low dilution rates but the level increased more rapidly with increasing dilution rate. The highest LDH levels were found in batch and continuous culture where the cells were growing on glucose at specific growth rates above 0.55 h^{-1} (Table 2, Fig. 5).

External phosphate concentration

The following experiments were performed to elucidate the effects of external inorganic phosphate on the *in vivo* behavior of *S.lactis* 65.1. In a batch culture growing on maltose the concentration of orthophosphate in the broth was suddenly raised by 100 mM. Following the phosphate addition the cells transiently increased their formate and decreased their lactate production rates. The yield of lactate was 39 % before, and 10 % during the first hour after the phosphate pulse. Three hours after the addition the lactate yield was back to 32 %. No increase in specific maltose consumption rate occurred after the phosphate pulse.

In a continuous maltose limited culture the transient periods following a pulse addition of maltose (10 $g \cdot l^{-1}$) and maltose (10 $g \cdot l^{-1}$) together with orthophosphate (100 mM) were studied by measuring the specific lactate production rate before and after the pulse addition of maltose (Fig. 6). Maltose added alone gave a more accelerated increase in specific lactic production more than when phosphate was administered together with maltose. The presence of phosphate resulted in more acetate and ethanol being formed during the transient period. These findings show that phosphate *in vivo* affects the rate of lactic acid production.

Studies were also performed in steady-state maltose-limited chemostats at two different orthophosphate concentrations

5 and 10 mM. The residual maltose concentrations and the maltose phosphorylase levels were similar in the two cultures showing that external phosphate did not affect maltose phosphorylase activity and the affinity of the cells for maltose. The lactate yields obtained were 60 % at 100 mM and 14 % at 5 mM phosphate, which was unexpected and indicated that intracellular phosphate concentration may vary.

DISCUSSION

In the glucose- and maltose plus glucose-limited cultures of S.lactis 65.1 the pattern of product formation showed a similar dependence on dilution rate as in S.lactis ML_3 (13). There was a marked difference between the maltose- and glucose-limited chemostats in that the former showed almost the same degree of heterofermentative product formation at all dilution rates.

The results indicated that S.lactis 65.1 formed acetate and ethanol from pyruvate either via the acetaldehyde-TPP complex (decarboxylation) or via pyruvate formate-lyase reaction (phosphoroclastic cleavage) releasing formate (Fig. 7). The regulation involved in the choice of pathway is not known. Pyruvate formate-lyase from S.feacalis is reportedly to be oxygen sensitive (9). The formation of formate in the aerated continuous culture of S.lactis 65.1 suggested the presence of a pyruvate formate-lyase. However, the specific formate production rate was lower than in the corresponding anaerobic culture.

The L-lactate dehydrogenase (L-LDH) from S.lactis 65.1 was subjected to the same type of regulation as that reported for L-LDH from other N-streptococci, where fructose 1,6-diphosphate (FDP) decreased the K_m value of L-LDH for pyruvate as well as NADH (7, 4, 12) (Fig. 7). The FDP activated L-LDH from S.lactis 65.1 was inhibited not only by orthophosphate but also by pyrophosphate. Orthophosphate was the stronger inhibitor of the two. The L-LDH was more susceptible to inhibition at low FDP concentrations indicating that phosphate and FDP were competing for the same enzyme site.

Thus the rate of lactic acid formation in S.lactis 65.1 may be influenced by the intracellular LDH, FDP, pyruvate, NADH and phosphate concentrations, internal pH and competing reactions (Fig. 7).

The LDH levels in cells from anaerobic maltose- and glucose-limited chemostats were similar in a wide range of dilution rates and the same LDH levels were found in galactose- and maltose-growing cells in batch culture. Measurements in batch cultures growing on glucose, maltose or galactose showed that the FDP pool was not affected by the carbohydrate used by S.lactis 65.1. Cells from chemostat cultures of S.lactis 65.1 were not assayed for their FDP contents. In S.lactis ML_3, however, the heterofermentative product formation in a glucose-limited chemostat culture was accompanied by a decrease in FDP concentration (13).

The much larger amounts of acetate and ethanol obtained in a maltose culture than in a glucose-limited culture cannot be explained by a smaller FDP pool or a lower LDH level. In batch culture the LDH level may be of some importance, but there must also be some other regulatory factor involved because cells growing on galactose or maltose in batch culture showed a different degree of heterofermentation (Table 2) with the same intracellular LDH and FDP levels.

Substantial differences were found between the *in vivo* measured lactate production rates and the calculated maximum possible rates *in vitro*. In batch and continuous culture on glucose the maximum *in vivo* rate was around 10 % of the calculated, indicating that the intracellular pyruvate or NADH concentrations were limiting as the FDP level should be enough for maximum LDH activity. Pyruvate concentrations of around 2 mM have been reported for *S.lactis* growing on glucose (16, 15). This concentration should allow over half the maximum LDH activity in the absence of phosphate and at optimal NADH concentration in *S.lactis* 65.1. Intracellular NADH and pyruvate thus appeared to limit the LDH activity of cells growing on glucose, assuming the absence of extreme deviations in internal pH from the extracellular pH. In a chemostat limited by maltose the maximum lactate production rate *in vivo* was only around 15 % of the maximum value found in corresponding glucose culture at the same LDH level.

S.lactis 65.1 was shown to have in inducible maltose

phosphorylase which required around 50 mM orthophosphate for maximum activity *in vitro*. Addition of phosphate to a growing culture did not increase the specific maltose consumption rate showing that the intracellular phosphate concentration was probably high enough to allow maximum maltose phosphorylase activity. In the presence of inorganic phosphate the L-LDH in *S.lactis* 65.1 and other N-streptococci (4, 7) increased its K_m value for pyruvate and NADH. When orthophosphate was extracellularily added as a pulse to the growing culture of *S.lactis* 65.1 the cells responded by decreasing their specific lactate production rate and increasing their acetate plus ethanol yield, which showed that phosphate influences product formation *in vivo*. Thus the internal inorganic phosphate concentration probably plays an important role in the regulation of LDH activity and product formation in *S.lactis* 65.1.

Pyruvate kinase from *S.lactis* (2, 3) was activated by FDP and this activation was inhibited by orthophosphate. Addition of 2 mM phosphate caused 80 per cent inhibition of the enzyme in the presence of 2 mM FDP showing that in N-streptococci phosphate appeared to be a stronger inhibitor of the pyruvate kinase than of the L-LDH activation. It is therefore probably that intracellular phosphate would also affect pyruvate kinase activity, possibly resulting in a decreased pyruvate pool. Phosphoenolpyruvate (PEP)was shown to accumulate in starved cells of *S.lactis* ML_3 cells. In these cells pyruvate kinase activators were exhausted and the pyruvate pool was

half that observed in glycolyzing cells (15).

Glucose is reportedly transported via the PEP-dependent phosphotransferase system in S.lactis (15). PEP transferase activity has also been found in S.lactis 65.1, whereas maltose is probably transported by a permease (M. Häggström, unpublished). It is possible that metabolism of glucose may affect the PEP pool and thereby the intracellular pyruvate concentration. The PEP pool in cells growing on different sugars remain to be measured.

It is suggested that in maltose metabolizing cells of S.lactis 65.1 the intracellular phosphate concentration is the main regulatory factor resulting in the pronounced heterofermentative product formation in batch as well as in continuous culture. The activation of LDH and probably also of pyruvate kinase are inhibited by orthophosphate. The regulation of LDH synthesis and the activity of the other enzymes in the pyruvate metabolism are not known.

In S.faecalis phosphate uptake is believed to require ATP (6). ATP is formed in both metabolic routes from pyruvate to acetate (Fig. 7). S.lactis 65.1 may need ATP for the active transport of phosphate to the maltose phosphorylase reaction and may thus have to choose a heterofermentative product formation, which may involve intracellular phosphate as a regulatory factor. In S.lactis 65.1 the steady-state lactate yield was higher at a low than at a high extra-

cellular phosphate concentration indicating that the need for phosphate transport may influence the intracellular phosphate concentration and the product formation.

In the presence of orthophosphate the maltose phosphorylase in S.lactis 65.1 cleaved the maltose molecule into glucose and β-glucose-1-phosphate. The enzyme appears to be of the same kind as those found in Neisseria meningitidis (5) and S.diacetylactis DRCI (11). It is not known how the reaction product β-glucose-1-phosphate formed enters the glycolytic pathway. Judging from the present findings energy in some form is necessary to convert β-glu-1P to a metabolite that can enter the glycolytic pathway.

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Table 1. Glucose-limited aerated culture of S.lactis 65.1. Glucose concentration in the substrate feed 10.0 $g \cdot l^{-1}$

Dilution rate (h^{-1})	L-lactate (mM)	Acetate (mM)	Formate (mM)	LDH activity ($U \cdot mg\ protein^{-1}$)	Residual glucose ($g \cdot l^{-1}$)	% C-recovery
0.10	56.6	24.8	10.9	8.0	0.06	73
0.26	73.4	9.5	2.2	10.2	0.06	74
0.38	71.2	6.5	1.1	12.9	2.4	93
0.56	46.6	5.7	< 1	14.1	5.4	102

Table 2. Fructose 1,6-diphosphate (FDP) pools and L-lactate dehydrogenase (L-LDH) levels in exponentially growing cells of S.lactis 65.1 from batch cultures on different sugars

Sugar	FDP (mM)	L-LDH (U/mg protein)	$Y_{EtOH + acetate}$ (%) [1)]
Glucose	13.6	19.9	< 2
Galactose	13.4	8.6	29
Maltose	15.9	9.2	64

1) expressed as percentage of theoretical values

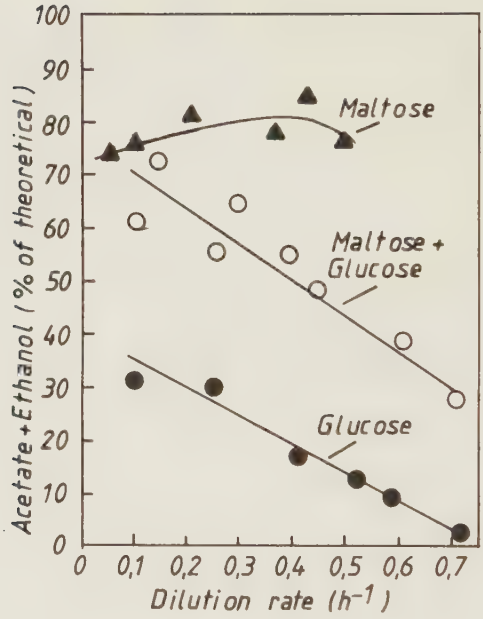

Figure 1 The yield of acetate plus ethanol in chemostat cultures of S. lactis 65.1 limited by different sugars.

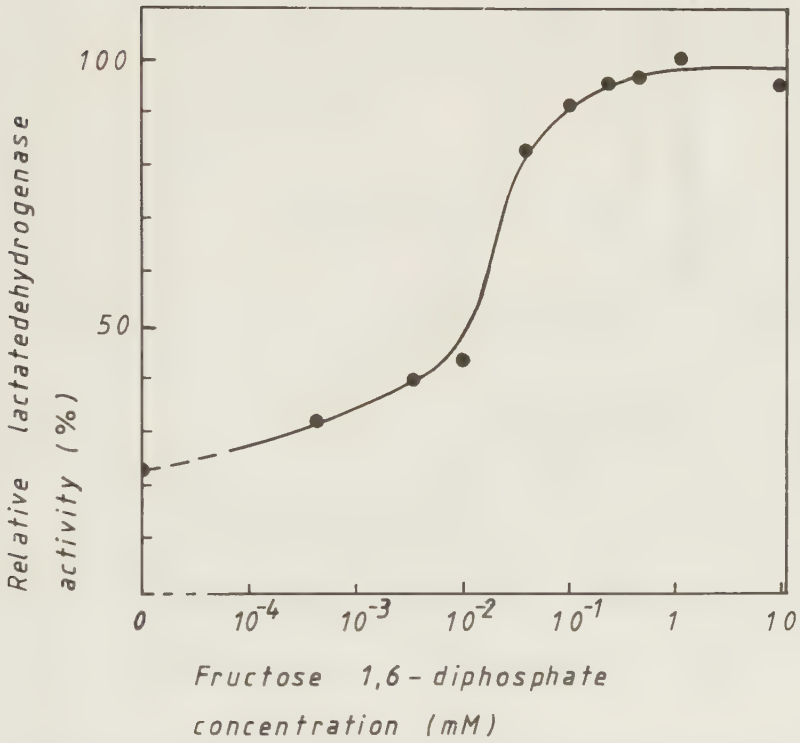

Figure 2 Relative activity in vitro of L-LDH from S. lactis 65.1 as a function of FDP concentration (LDH assayed as described in Material and Methods)

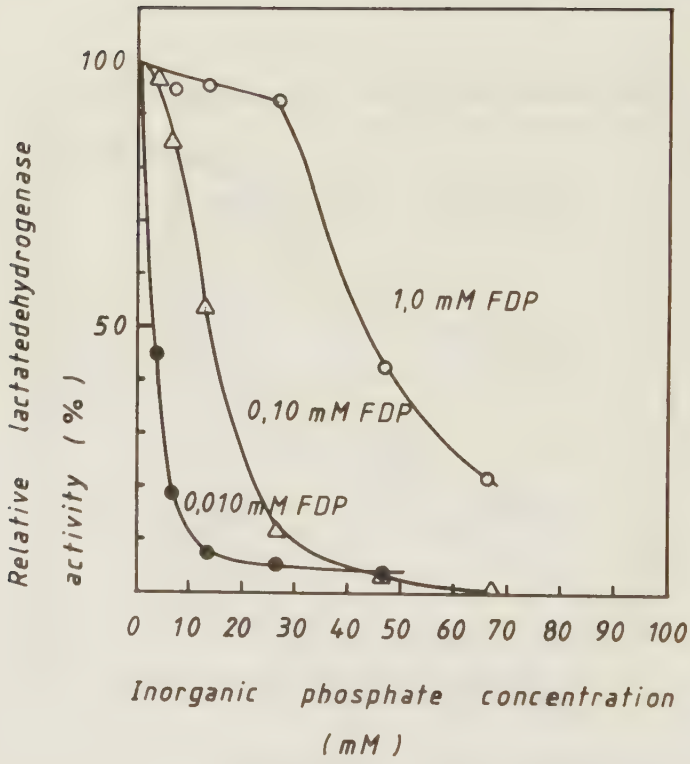

Figure 3 Relative in vitro acitivity (at each FDP level) of L-LDH from S. lactis 65.1 as a function of orthophosphate concentration (L-LDH assayed as described in Material and Methods).

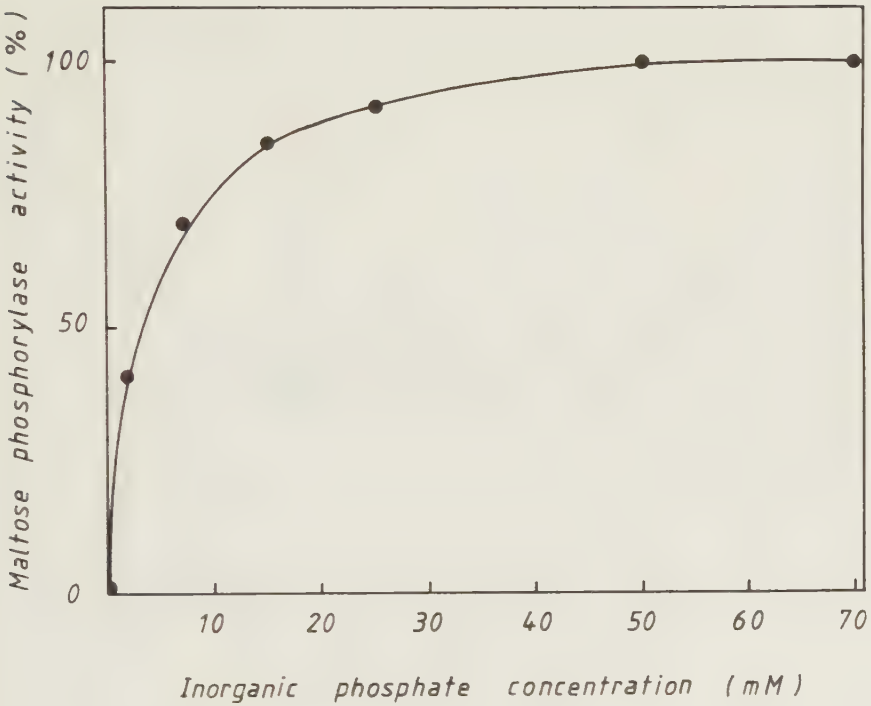

Figure 4 Activity in vitro of maltose phosphorylase (assayed as described in Material and Methods) from S. lactis 65.1 as a function of orthophosphate concentration. The activity expressed relative to maximum.

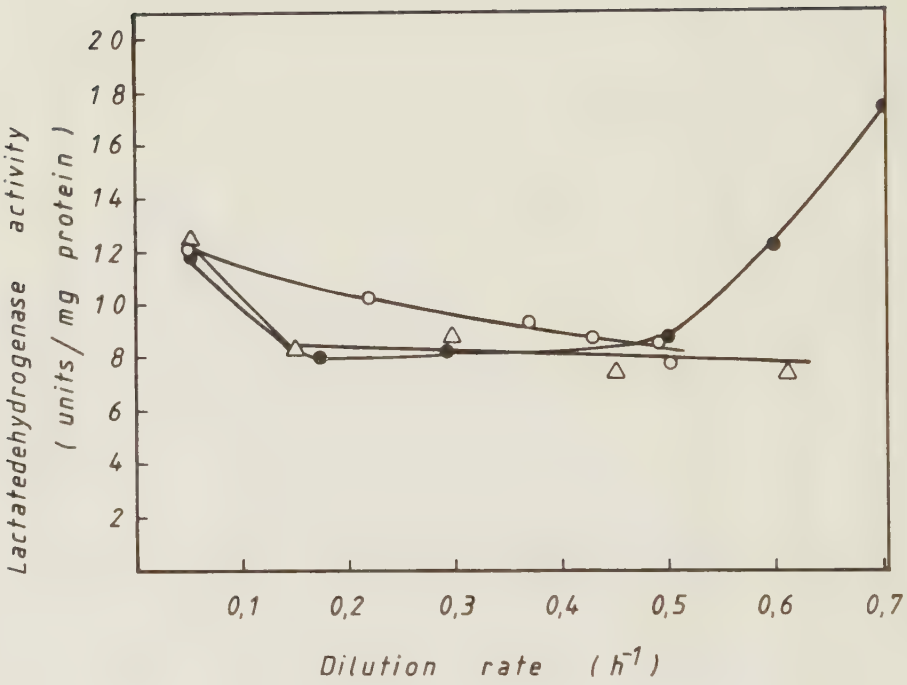

Figure 5 Lactate dehydrogenase activity in cells of S. lactis 65.1 growing in chemostat cultures limited by glucose (- ● -), maltose (- ○ -) or maltose and glucose (- △ -).

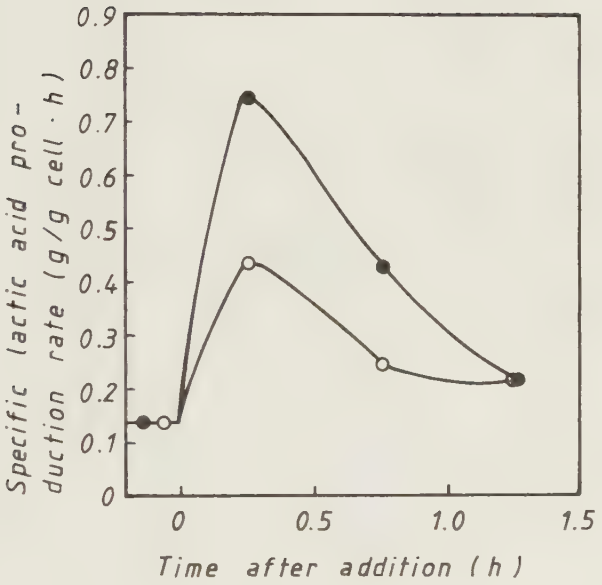

Figure 6 Transient response of a maltose-limited culture of S. lactis 65.1 after a pulse addition (indicated by arrow) of maltose (10 $g \cdot l^{-1}$) (- ● -) and maltose (10 $g \cdot l^{-1}$) plus ortophosphate (100 mM) (- ○ -). Dilution rate 0.20 h^{-1}.

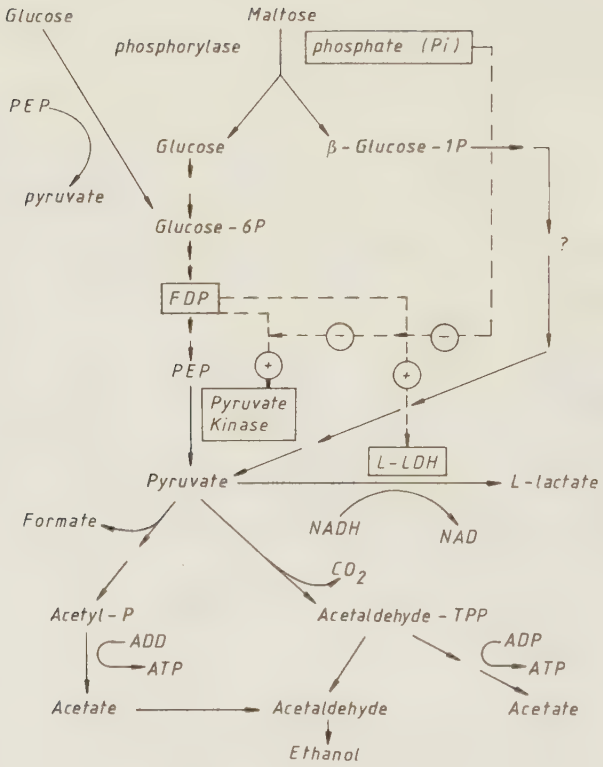

Figure 7 Schematic representation of carbohydrate catabolism in S. lactis 65.1 and the regulatory factors involved. Fructose 1,6-diphosphate (FDP), phosphoenolpyruvate (PEP), L-lactate dehydrogenase (L-LDH).

IV

Production of L- and D-lactate by Pediococcus pentosaceus in batch and steady-state continuous culture.

M.H. Häggström and E. Andersson

SUMMARY

A strain of Pediococcus pentosaceus, which is used as starter culture in the production of dry sausages, was dependent on the presence of acetate for fast growth. In a batch culture on a mixture of glucose and sucrose both sugars were consumed simultaneously. Similar growth rates and product yields were obtained on glucose and sucrose. D- and L-lactic acid were formed by a D- and a L-lactate dehydrogenase, respectively, and no racemase was present. In batch cultures around 15 % of the lactic acid produced was the D-isomer, whereas in a sucrose-limited continuous culture the fraction of D-lactic acid increased with decreasing dilution rate. The results are discussed in relation to the two LDH acitivities.

INTRODUCTION

All lactic acid bacteria produce lactic acid as one of the products of carbohydrate metabolism. Pediococci are regarded as homofermentative organisms and racemic lactic acid is the main product of glucose fermentation (Kitahara 1974).

The two isomers of lactic acid produced by lactic acid bacteria can be formed by D- and L-lactate dehydrogenases (LDH) or via L-LDH and a racemase (Kunath & Kandler 1980). Pediococcus cerevisiae was reported to contain L- and D-lactate dehydrogenases with different pH optima and the organism produced a mixture of L- and D-lactic acid in a batch culture without pH control (Gordon & Doelle 1975).

Reports indicate that P.lindneri and P.hennebergi also have a D- and a L-LDH (Hiyama et al. 1968). L-lactate dehydrogenase from N-streptococci was activated by fructose 1,6-diphosphate (FDP), and the activated LDH was inhibited by orthophosphate (Crow & Pritchard 1977; Jonas et al 1972). N-streptococci reportedly possess a single LDH, which is specific for L-lactic acid. S.lactis 760, however, is an exception as it has both a L- and a D-lactate dehydrogenase, and only the L-LDH is activated by FDP (Mou et al. 1972). As pediococci and streptococci belong to the same family their L-lactate dehydrogenases may be expected to be regulated in a similar way. The effect of FDP on the acitivity of LDH from pediococci is not known. The present investigation was undertaken to study the effects of growth conditions on product formation and substrate utilization in P.pentosaceus. The enzymes involved in the production of L- and D-lactic acid and their regulation in this organism were also studied. P.pentosaceus is used as starter culture in the production of dry sausages.

MATERIALS AND METHODS

Organism

A strain of Pediococcus pentosaceus, which is used as the starter culture "Culcid" was obtained from the Swedish Meat Research Institute, Kävlinge, Sweden. It was stored in lithmus milk at -20^{o} C.

Medium

The medium used in batch and continuous fermenter cultivations and for growing inocula contained in $g \cdot l^{-1}$: Tryptone 5.0, yeast extract 20.0, casamino acids 4.0 (all products of Difco Laboratories), K_2HPO_4 2.5, KH_2PO_4 2.5, $MgSO_4 \cdot 7H_2O$ 0.5, $MnSO_4 \cdot H_2O$ 0.1, and Na-acetate 1.5 unless otherwise stated. For the fermenter cultivations sucrose and/or glucose were added as indicated in the text, and 20 $g \cdot l^{-1}$ sucrose or glucose was used for the inoculum preparation.

Growth conditions and measurements

The batch and continuous fermentations were performed in fermenters with working volumes of 0.7 and 1.0 litres. Unless otherwise stated, the temperature and pH were maintained at 40° C and 6.5, respectively. Anaerobic conditions were maintained by a flow of N_2:CO_2 (95:5) through the head-space of the fermenter. Growth was followed as optical density (OD) in a Turner photometer at 620 nm. Cell dry weight was determined by centrifuging a defined volume of cell-suspension, washing of the pellet and drying at 105° C for 12 h.

Analyses of carbohydrates and products

The cells in the samples from the fermenter were rapidly removed by centrifugation and membrane filtration, and the supernatant was analysed for substrates and products. Glucose was assayed with a hexokinase/glucose 6-phosphate based kit

and sucrose was assyed as glucose following a hydrolytic step using β-fructosidase. L-lactate was measured with L-lactate dehydrogenase based kit and D-lactate was determined in the same way but with the use of D-lactate dehydrogenase (all products of Boehringer, Mannheim, Germany). Acetic acid and ethanol were assayed on a Varian 940 gas chromatograph equipped with a 1/16 inch I.D., 8 feet long teflon column containing Chromosorb 101. The column temperature was 160° C and nitrogen saturated with formic acid was used as carrier gas with a flow rate of 30 ml·min^{-1}.

Cell-free extract

Thirty millilitres of cell suspension was removed from the culture, centrifuged for 10 min at 15,000 g, 4° C, and washed with 0.04 M sodium phosphate buffer pH 6.8. The pellet was suspended in 3 ml of the same buffer, 2.5 ml glass beads (0.2 mm) were added, and the cells were disintegrated for 3 minutes on ice in an ultrasonic disintegrator (MSE, Crawley, England). Cell debris was removed by centrifugation at 30,000 g, 4° C for 30 min. The supernatant was used as cell-free extract. Protein concentration in the extract was measured according to the method of Lowry et al. (1951) using bovine serum albumin fraction V (Boehringer) as standard.

Enzyme activities

Sucrose hydrolyzing activity was assayed qualitatively in the cell-free extract. The assay mixture contained 0.16 M sucrose,

0.1 M sodium citrate or sodium phosphate buffer pH 6.5 and crude extract. The reaction temperature was 30° and glucose released was measured as described above.

Racemizing activity was determined qualitatively in cell-free extract. The assay system contained 4.0 mM sodium phosphate buffer, 13 mM L-lactate and cell-free extract. An aliquot was taken from the assay system at different intervals and the reaction was stopped by heating to 90° C. D-lactate was determined according to the method described above.

The total lactate dehydrogenase activity was measured as pyruvate reduction in a 3 ml assay system containing 67 mM tris (hydroxymethyl)aminomethane hydrochloride pH 7.0, 0.16 mM NADH, 33 mM sodium pyruvate and cell-free extract. Fructose 1,6-diphosphate tetracyclohexylammonium salt (FDP) was added to the assay mixture when indicated. The reaction was run at 25° C and one unit of enzyme activity was defined as the amount of enzyme oxidizing 1 µmole NADH/min. When pyruvate was omitted from the assay system no NADH oxidase activity was detected. Lactate oxidation was used for measuring the separate D- and L-lactate dehydrogenase activities. The assay system contained 67 mM tris (hydroxymethyl)aminomethane hydrochloride pH 8.0, 4.8 mM NAD, 51 mM L-glutamate, 102 mM D- or L-lactate, glutamate-pyruvate-transaminase (GPT) 2.4 U/ml, and cell-free extract, and was run at 25° C. One unit of enzyme activity was defined as the amount of enzyme reducing 1 µmole NAD/min.

Calculations

From the data obtained in the fermenter cultivation the following parameters were calculated. Yield coefficient (mole/mole) ($Y_{p/s}$) was expressed as a fraction of the theoretical value on the actual substrate. Specific lactate production rate ($q_{lactate}$) in continuous steady-state culture was $(D \cdot P) \cdot X^{-1}$, where D denotes the dilution rate (h^{-1}), P the lactate concentration ($g \cdot l^{-1}$) and X the cell dry weight ($g \cdot l^{-1}$). The specific growth rate (μ) and the substrate concentration (K_s) required for half maximum specific growth rate were estimated from continuous culture data by using the equation $\frac{1}{\mu} = \frac{1}{s} \quad \frac{K_s}{\mu_{max}} + \frac{1}{\mu_{max}}$ where s denotes the residual substrate concentration and $\mu = D$.

RESULTS

Strain characteristics

P.pentosaceus fermented glucose, sucrose and mannitol, but no acid was produced from maltose, xylose or cellobiose. The organism required a rich complex medium for growth. Flask experiments showed that acid production measured as a pH change, was faster at 40° C than at 30° C and acetate increased the acid production rate (Fig. 1).

Batch cultures

Batch cultivations were studied at a constant pH on glucose,

sucrose or a mixture of sucrose and glucose. When P.pentosaceus fermented sucrose, D- and L-lactate were the main products (Fig. 2). No ethanol or acetic acid production occurred, and the amount of sodium hydroxide added corresponded to the total amount of lactic acid produced. Similar data were obtained when glucose was fermented. P.pentosaceus simultaneously fermented both sugars at the same rate in a batch culture on a mixture of glucose and sucrose (Fig. 3). D-lactate concentration was 2.2 $g \cdot l^{-1}$ after 6 hours cultivation. There was no change in L-lactate concentration when this culture was left for 16 h after all sugar had been consumed. The total overall lactate yield in the batch cultures on glucose, or sucrose alone or on a mixture of the two sugars was 88-91 % and D-lactate constituted 14-16 % of it.

Continuous culture

The product formation and substrate utilization by P.pentosaceus was studied in a continuous sucrose-limited chemostat culture (Fig. 4). The sucrose concentration in the medium was 14.4 $g \cdot l^{-1}$. The D-lactate proportion of the total lactate produced decreased with increasing dilution rate. The specific L-lactate production rate increased with the dilution rate, whereas the specific rate of D-lactate formation was fairly constant in the dilution rate range 0.20 - 0.75 h^{-1} (Table 1). Small amounts of acetic acid were produced at low dilution rates. The carbon balance at a dilution rate of 0.06 h^{-1} suggests formation of additional unidentified products. From the data obtained in the continuous culture K_s

for sucrose and μ_{max} were estimated to be 0.33 $g \cdot l^{-1}$ and 0.80 h^{-1} respectively.

Enzyme activities

Cells of P.pentosaceus grown on glucose were subjected to disintegration and sucrose hydrolyzing activity was found in the cell-free extract. Cell-free extracts prepared from P.pentosaceus showed no racemazing activity. Fructose 1,6-diphosphate (FDP) and orthophosphate had no effect on the activity of lactate dehydrogenase (LDH) from P.pentosaceus (Table 2). The LDH activity at pH 5.0 was slightly lower than at pH 7.0.

The two separate lactate dehydrogenase activities were identified in cell-free extract of P.pentosaceus from a batch culture on sucrose. L- and D-LDH activities constituted 86 and 15 per cent respectively of total activity. Lactate oxidation was carried out at pH 8.0, at which both enzymes showed maximal activity. At pH levels below 7 the activities were substantially lower.

DISCUSSION

Pediococcus pentosaceus required acetate in the medium for fast growth indicating that the organism can supply itself with acetate or acetyl-CoA only at a limited rate. These findings were confirmed in continuous culture, where acetate was accumulated at low, and was consumed at high,

dilution rates.

In P.pentosaceus sucrose was probably hydrolysed intracellularly to glucose and fructose. When growing on a mixture of glucose and sucrose both sugars were consumed simultaneously at the same rate and both sugars resulted in the same product formation.

L- and D-lactic acid were produced via a L- and a D-lactate dehydrogenase, respectively, and no racemase was present. These results are comparable to those found for P.cerevisiae (Gordon & Doelle 1975). In P.cerevisiae, however, D-lactic acid formation started below pH 6.0 whereas at pH 6.5 D-lactate was produced by P.pentosaceus. The ratio of D- to L-lactic acid formed in batch culture was in agreement with the in vitro measured ratio of D- to L-lactate dehydrogenase activity. These results, and the fact that the L-lactic acid concentration in the broth did not change when the culture was left at constant pH for 16 hours, strongly indicate that the two isomers are formed directly in association with the sugar catabolism. This means that there was no flow of L-lactate formed via pyruvate to D-lactate.

The results of the sucrose-limited continuous culture showed that D-LDH activity was constant in a wide range of dilution rates, whereas L-LDH activity increased with the dilution rate. Increased activity of existing L-LDH or a higher L-LDH level are the two possibilities by which the cell can increase its LDH activity. In N-streptococci the L-LDH was activated

by fructose-1,6-diphosphate but not LDH from P.pentosaceus. In S.lactis grown in a glucose-limited chemostat the FDP and L-LDH levels in the cells increased with the dilution rate and the observed change in product formation could be explained by activation as well as by a higher level of L-LDH (Thomas 1979). It therefore appears likely that in P.pentosaceus the L-LDH, but not the D-LDH, level was rising with the dilution rate in continuous culture. However, the presence of an additional activator of L-LDH from P.pentosaceus cannot be ruled out.

Acknowledgements: This work was supported by a grant from The Swedish Biotechnology Foundation and The Swedish Board for Technical Development.

Table 1

Parameters from a continuous sucrose-limited culture of P.pentosaceus

D (h^{-1})	X ($g \cdot l^{-1}$)	$q_{L\text{-lactate}}$ ($g \cdot g^{-1} \cdot h^{-1}$)	$q_{D\text{-lactate}}$ ($g \cdot g^{-1} \cdot h^{-1}$)	$Y_{L\text{-lactate}}$ (%)	$Y_{D\text{-lactate}}$ (%)	$Y_{acetate}$ (%)
0.06	2.20	0.22	0.12	53	29	4
0.20	2.52	0.80	0.32	67	27	2
0.35	3.14	1.17	0.36	71	22	C
0.53	3.15	1.88	0.44	76	18	C
0.64	2.77	2.50	0.36	80	12	C
0.68	2.78	2.59	0.37	81	12	C
0.75	2.54	2.94	0.31	88	9	C

C = acetate consumed

Table 2

Relative activity, measured as pyruvate reduction rate, of both lactatedehydrogenases from P.pentosaceus

FDP mM	Ortho-phosphate	pH	% activity
0	0	7.0	100
0.001	0	7.0	103
0.01	0	7.0	100
10	0	7.0	94
10	33	7.0	97
0	0	5.0[1)]	86

1) 67 mM sodium citrate buffer instead of TRIS-buffer in the assay system

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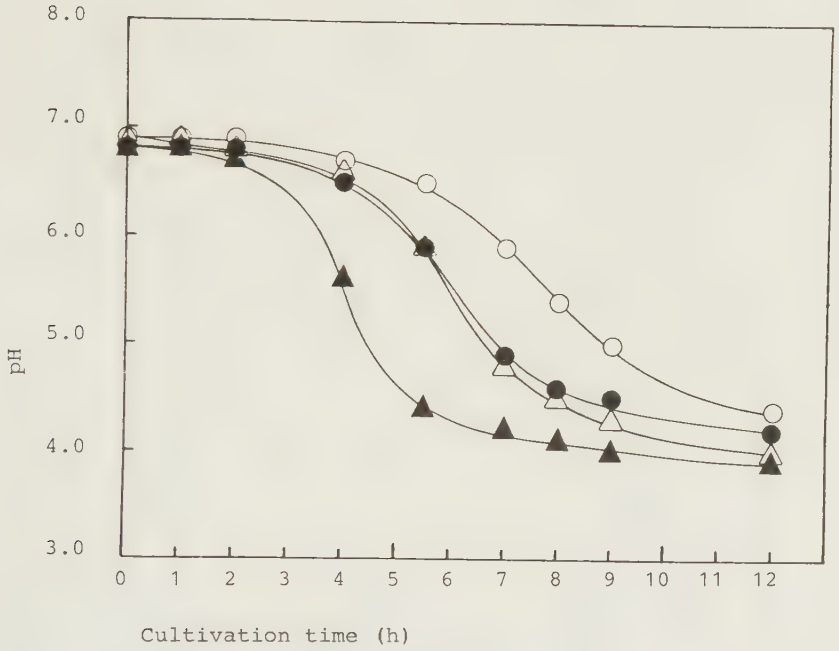

Figure 1 Acid production (measured as pH change) by P. pentosaceus at 40° C with acetate (- ▲ -), no acetate (-△-) and at 30° C with acetate (- ● -), no acetate (- ○ -).

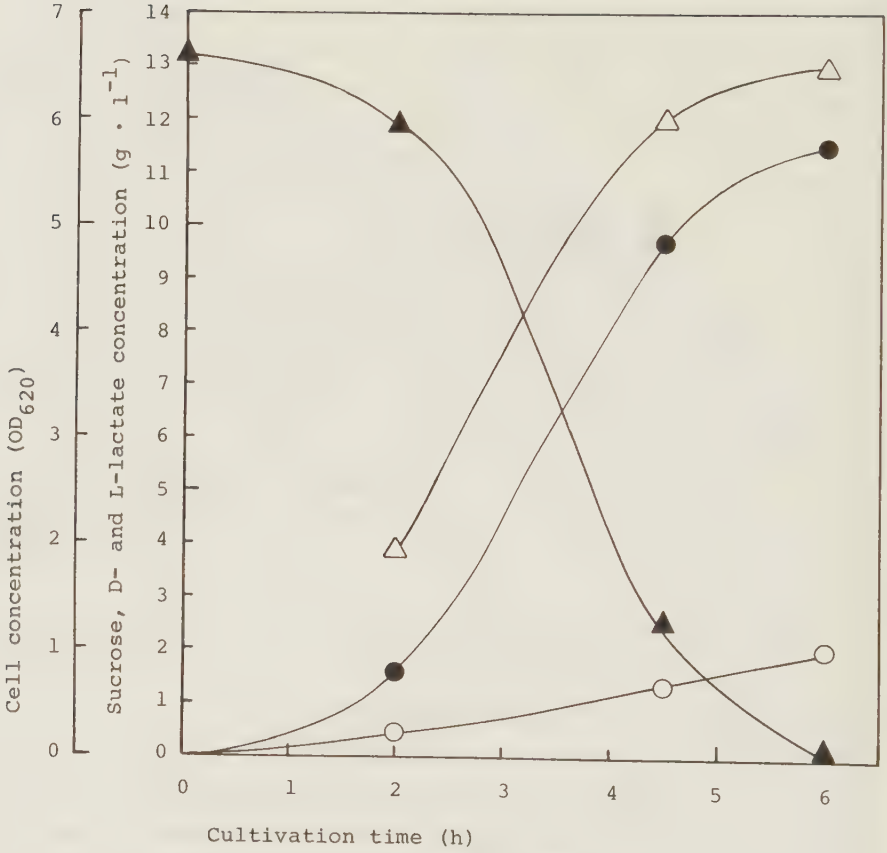

Figure 2 Growth characteristic of P. pentosaceus in batch culture on sucrose (13.2 $g \cdot l^{-1}$). Sucrose (- ▲ -), D-lactate (- ○ -), L-lactate (- ● -) concentrations in $g.l^{-1}$ and cell concentration (- △ -) in OD (620 nm).

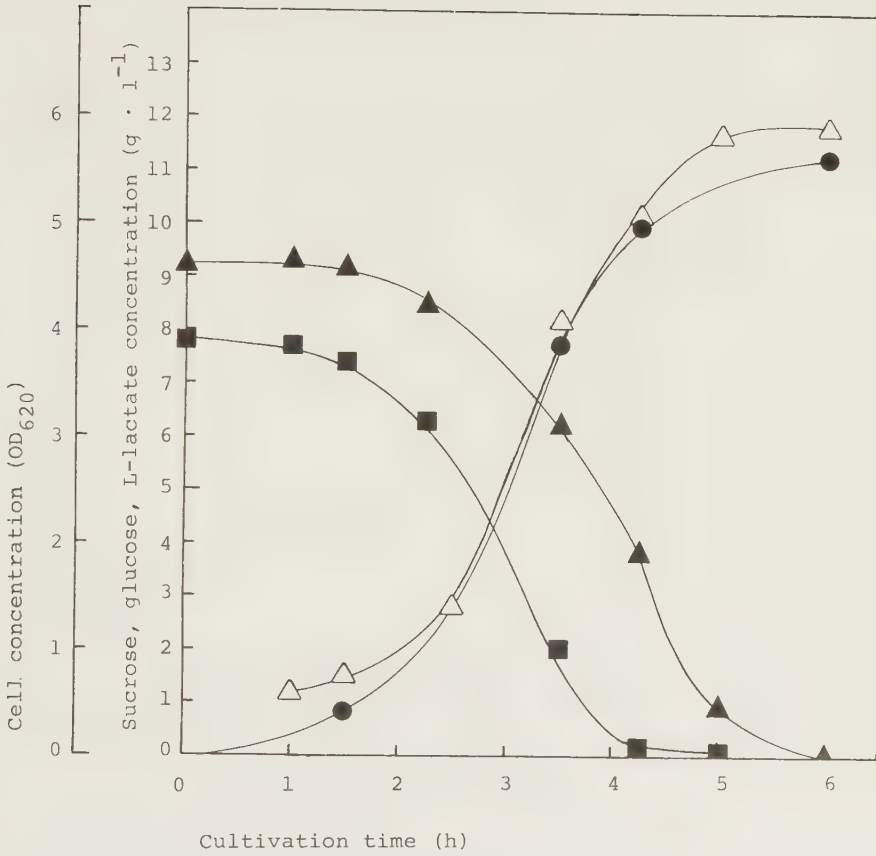

Figure 3 Batch culture of P. pentosaceus on a mixture glucose (7.8 $g \cdot l^{-1}$) and sucrose (9.2 $g \cdot l^{-1}$). Sucrose (- ▲ -), glucose (- ■ -), L-lactate (- ● -) concentrations in $g \cdot l^{-1}$ and cell concentration (- △ -) in OD (620 nm).

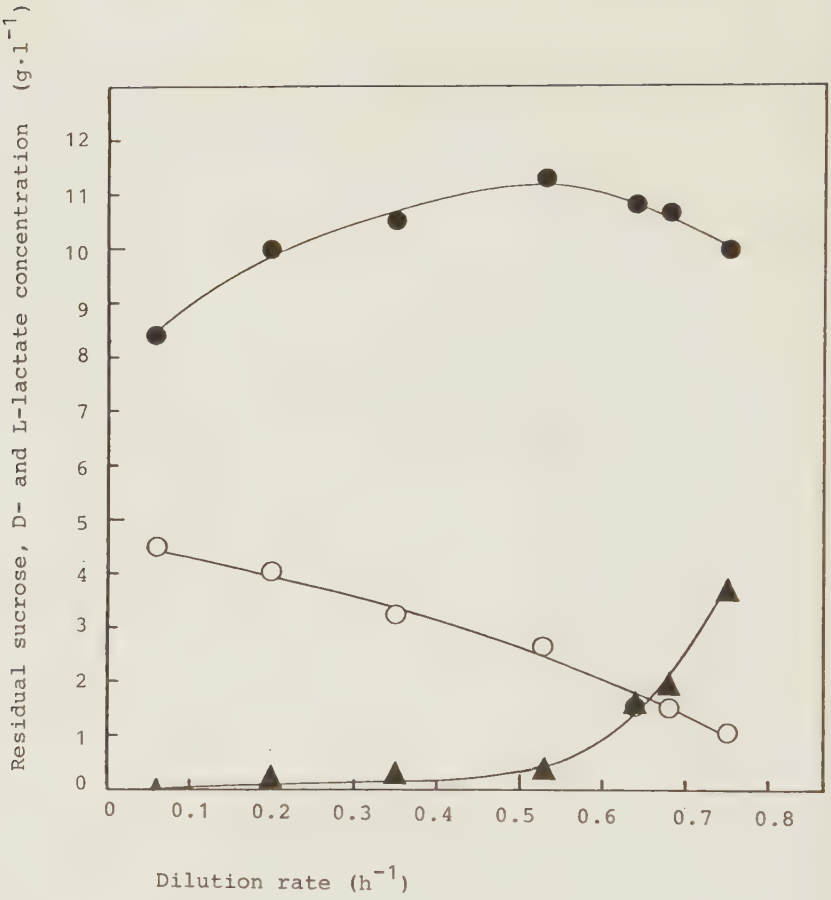

Figure 4 Continuous sucrose-limited culture of P. pentosaceus. Residual sucrose (- ▲ -), D-lactate (- ○ -), and L-lactate (- ● -) concentrations in $g \cdot l^{-1}$.

Regulation of a mixed culture of *Streptococcus lactis* and *Saccharomycopsis fibuliger*.

M.H. Häggström and M. Dostâlek

SUMMARY

In a mixed culture of Saccharomycopsis fibuliger Y76 and Streptococcus lactis 65.1 on starch the main interactions are commensalism and competition. Oxygen-limited batch and continuous cultures of S.fibuliger showed accumulation of sugars. When oxygen was used as an external regulatory parameter in the mixed culture lactic acid, acetic acid and formic acid were formed. Ethanol produced by S.lactis was most likely assimilated by S.fibuliger. Continuous mixed cultures were stable under conditions of oxygen limitation at the dilution rates tested (0.10 h^{-1} and 0.15 h^{-1}). Conversion yields of 35 to 40 % were obtained but may be improved.

INTRODUCTION

Interactions between two microbial species in mixed cultures may be of different types such as commensalism, mutualism and competition (Harrison 1978). One of the difficulties in using mixed cultures industrially is the instability of most systems especially in continuous steady-state operation. In a mixed continuous culture stability can be secured when the product of the first species is the growth limiting substrate for the other organism (commensalism) (Lee et al. 1976). Mixed continuous cultures based on pure mutualism are instable but may stabilize when component species are competing

for a third substrate (Meyer et al. 1975). A system where the organisms are competing for the same growth-limiting substrate is unstable. Double substrate-limitation in a competing system may lead to stable steady-state dilution rate regions (Yoon and Blanch 1977).

Streptococcus lactis belonging to the N streptococci is regarded as homofermentative, and is used in the dairy industry, where it produces lactic acid from lactose in milk. These organisms are fermentative but aerotolerant. In batch culture S.lactis 65.1 ferments glucose to lactic acid, whereas the products formed from maltose are acetic acid, ethanol and formic acid in addition to lactic acid. Glucose and maltose are sequentially metabolized in batch culture, whereas both are utilized simultaneously in continuous carbohydrate-limited cultures (Häggström). Saccharamycopsis fibuliger (Endomycopsis fibuliger) is an aerobic organism which can hydrolyze starch.

This report concerns a mixed culture system of S.lactis and S.fibuliger, where the main interactions are commensalism and competition. The aim of the investigation was to stabilize and regulate the composition of the mixed culture by limiting the supply of oxygen and to study the product formation.

MATERIALS AND METHODS

Organisms

Streptococcus lactis 65.1 from the Swedish Dairies Association, Malmö, Sweden was stored in lithmus milk at -20° C. Saccharomycopsis fibuliger NRRL Y76 was kept on yeast extract peptone agar at + 4° C and was transferred every third week.

Media

The medium used for fermenter cultivations of S.fibuliger in monoculture contained in $g \cdot l^{-1}$: yeast extract 5.0, K_2HPO_4 2.5, KH_2PO_4 2.5, $MgSO_4 \cdot 7H_2O$ 0.5, $(NH_4)_2SO_4$ 5.0 and soluble starch 15.0. For growing inocula the same medium was used, but with 10 $g \cdot l^{-1}$ glucose instead of starch. In the mixed culture fermenter cultivations the monoculture medium described above was used, but with the addition of tryptone 5.0 $g \cdot l^{-1}$ and casamino acid 1.0 $g \cdot l^{-1}$. Inocula of S.lactis was grown on the mixed culture medium without $(NH_4)_2SO_4$ and starch, but with the addition of maltose 20.0 $g \cdot l^{-1}$.

Growth conditions and measurements

Fermenters with working volumes of 2.5 and 0.7 litres (Chemoferm, Hägersten, Sweden) were used for the batch

and continuous cultivations. The pH was kept constant at 6.5 with the aid of sodium hydroxide and the temperature was 30° C. The volume in the continuous culture experiments was kept constant with a conductivity type level controller.

Inocula for the batch mixed cultures were prepared by growing the two component strains separately and mixing them on inoculation. The continuous mixed cultures were started as a continuous monoculture of S.fibuliger. When steady-state conditions had been reached 20 % of the culture was pumped out and replaced by S.lactis from a batch culture grown on maltose.

The rate of cell growth was followed by measuring culture turbidity in a Turner photometer at 620 nm. The dry weight of the cells in the monocultures was determined by centrifuging a cell suspension, washing the pellet, and drying it for 12 h at 105° C.

Analyses of substrate and products

The presence of starch was qualitatively tested with Lugol's solution. Cells in the samples from the fermenter were rapidly removed by centrifugation and membrane filtration and the supernatants were analysed. Reducing sugars were determined according to the method described by Miller et al. (1960) and the

values were expressed as g glucose equivalents. Glucose was measured with a hexokinase/glucose 6-phosphate kit (Boehringer Mannheim, Germany). Maltose was assayed as glucose following a hydrolytic step using α-glucosidase (Sigma Chemical Co, Mo, USA). A L-lactate dehydrogenase kit was used for measuring L-lactate (Boehringer). Formate was assayed according to the method of Lang and Lang (1972) using citric acid. Acetic acid and ethanol were determined on a Varian 940 gas chromatograph equipped with a 1/16 inch I.D. 8 feet long teflon column containing Chromosorb 101. The column temperature was 160° C and nitrogen saturated with formic acid was used as carrier gas at a flow rate of 30 $ml \cdot min^{-1}$.

RESULTS

Monoculture of S.fibuliger

S.fibuliger was grown in batch and continuous culture on starch, and the sugar released into the medium under different growth conditions was measured. In a batch culture where aeration was turned off after a certain time during the cultivation an accumulation of sugar, measured as glucose, and a limited cell growth were obtained (Fig. 1). The air supply was stopped while the starch reaction was still positive. The overall glucose production rate after aeration had been stopped was 0.8 $g \cdot l^{-1} \cdot h^{-1}$.

The accumulation of sugars following a limited supply of oxygen to the culture was studied also in continuous cultures. At a constant oxygen transfer rate the production of sugars increased with the dilution rate while the steady-state cell concentration decreased (Fig. 2). In this experiment glucose, maltose and reducing sugars were measured. Residual starch and maltose appeared in the broth at the higher dilution rate. At the highest dilution rate the productivity and specific production rate of reducing sugars obtained were 1.5 $g \cdot l^{-1} \cdot h^{-1}$ and 1.7 $g \cdot g\ cell^{-1} \cdot h^{-1}$, respectively.

The results obtained thus show that sugar may accumulate under oxygen-limiting conditions and that the relative amounts of individual sugars vary with the dilution rate.

Batch cultures of S.lactis and S.fibuliger

Mixed cultures in which S.fibuliger is producing fermentable sugars for S.lactis under oxygen-limited conditions were studied (Fig. 3). In a batch culture where a constant, low supply of oxygen was maintained throughout the cultivation there was an initial slow increase in reducing sugars followed by a rapid accumulation when the oxygen supply limited the growth of the aerobic S.fibuliger (Fig. 4). Heterofermentative product formation, represented by acetate and lactate occurred and no ethanol was detected in the medium indicating

that both glucose and maltose were fermented by S.lactis. Another batch culture was performed where aeration was turned off and nitrogen was added to ensure anaerobic conditions. Product formation during the anaerobic period is summarized in Table 1. In this case ethanol was formed. In shake flask experiments acetate and ethanol were assimilated by S.fibuliger. These results indicate that ethanol formed by S.lactis in the mixed culture was consumed by S.fibuliger in the presence of oxygen.

Continuous mixed culture

S.fibuliger and S.lactis were grown in continuous culture at two different oxygen transfer rates which was effected by changing the aeration rate at a constant stirrer speed (Table 2). Ethanol was present in concentration lower than 0.15 $g \cdot l^{-1}$. As maltose and glucose are the only sugars fermented by S.lactis the total product yields obtained showed that the degree of starch hydrolysis was higher at the higher oxygen transfer rate.

We studied the stability of a continuous mixed culture under oxygen-limited conditions for 50 hours. Product concentration and residual reducing sugars were measured (Fig. 5). The fluctuations observed are probably due to difficulties in keeping the aeration rate constant at

the low gas flow rates used. Formate concentrations varied between 1.5 and 1.7 $g \cdot l^{-1}$. When the dilution rate in this culture was decreased from 0.15 to 0.10 h^{-1} the new steady-state product formation changed with an increase in the amount of acetate and a decrease in the amount of lactate but the total yield increased from 0.35 to 0.40 g products per g starch, showing that S.lactis was fermenting more maltose at the lower dilution rate.

DISCUSSION

The interactions involved in the mixed culture of S.lactis 65.1 and S.fibuliger are illustrated in Fig. 3. The main interactions can be characterized as commensalism and competition. The second organism, S.lactis, is dependent on the first organism S.fibuliger for the production of fermentable sugars. Both organisms are competing for the sugars produced, which are converted to cell mass and carbon dioxide by S.fibuliger or fermented to lactic acid, acetic acid, formic acid and ethanol by S.lactis. When S.fibuliger was grown in batch and continuous oxygen-limited cultures reducing sugars accumulated in the medium. Oxygen supply rate was therefore chosen as a regulatory agent in the mixed cultures of S.fibuliger and S.lactis.

Maltose results in a hetero- and glucose in a homo-

fermentative product pattern in batch cultures of S.lactis 65.1. Glucose completely inhibits utilization of maltose (Häggström). In an oxygen-limited batch culture of S.lactis and S.fibuliger both lactate and acetate accumulated indicating that the fermentable substrate available to S.lactis was a mixture of maltose and glucose, and that glucose must be present in limiting concentrations.

Results obtained in the continuous mixed cultures showed that the oxygen supply rate and the dilution rate affected the relative amounts of the products formed. This behaviour may be explained by the dependence of heterofermentation in S.lactis on the dilution rate (Thomas 1979) and carbon source fermented (Häggström). When the oxygen supply rate is increased at a constant dilution rate or when the dilution rate is decreased at a constant oxygen supply rate the cell concentration of the aerobic S.fibuliger is expected to increase. Under both these conditions the total product yield increased indicating that the conditions were not optimized for a maximal product yield. Judging from the results the stability of this externally controlled mixed culture in continuous operation was good.

Ethanol produced by S.lactis can be assimilated by S.fibuliger. In the anaerobic mixed batch culture ethanol formed and thereby suggesting that ethanol was

assimilated by S.fibuliger in the presence of oxygen in the mixed culture. In Endomycopsis bispora glucoamylase is inhibited by glucose (Ruttloff et al. (1979) and in S.fibuliger glucose mediates catabolite repression of glucoamylase synthesis (Afanasyeva 1978). The presence of a second organism consuming glucose would release the inhibition and/or repression by glucose and thereby increase the specific sugar production rate of the first organism.

In the continuous monoculture of S.fibuliger maltose did not accumulate at the lower dilution rates, whereas in the continuous mixed culture the product yields showed that maltose was fermented by S.lactis. In the mixed culture the two organisms are competing for maltose and the results obtained indicate that S.lactis has a higher affinity for maltose than has S.fibuliger.

In a mixed culture starch was made available as a substrate for S.lactis without the use of a prehydrolyzing step. Mixed culture systems may be considered an alternative to genetic modifications of organisms. The method described for controlling the composition of a mixed culture by the external regulator, oxygen, may be applied to systems where component species are competing for a common substrate and one of the organisms is dependent on oxygen for growth and the other is aerotolerant.

Acknowledgements. This work was supported by a grant from The Swedish Biotechnology Foundation and The Swedish Board for Technical Development. The technical assistance of Miss I. Paulsson was invaluable.

Table 1. Products formed and reducing sugars consumed during the anaerobic part of a batch mixed culture of *S.lactis* 65.1 and *S.fibuliger* Y76

Reducing sugars ($g \cdot l^{-1}$)	L-lactate ($g \cdot l^{-1}$)	Acetate ($g \cdot l^{-1}$)	Etanol ($g \cdot l^{-1}$)	Formate ($g \cdot l^{-1}$)
3.95	1.4	0.85	0.60	0.90

Table 2. Concentrations of intermediates and products in a continuous mixed culture of S.fibuliger and S.lactis at different aeration rates (VVM) and constant stirrer speed. Dilution rate was 0.20 h^{-1}. Starch in the medium feed 15 $g \cdot l^{-1}$

VVM	Reducing sugar ($g \cdot l^{-1}$)	L-lactate ($g \cdot l^{-1}$)	Acetate ($g \cdot l^{-1}$)
0.05	3.3	3.7	1.04
0.10	1.6	5.7	0.46

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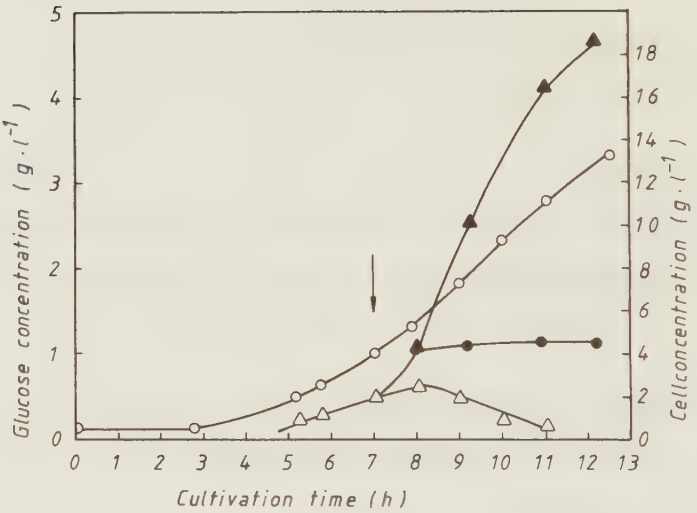

Figure 1 Glucose accumulation in batch cultures of S. fibuliger Y76. At the time indicated by the arrow aeration was turned off during one cultivation. Glucose (- △ -) and cell (- ○ -) concentration in the aerated culture and glucose (- ▲ -) and cell (- ● -) concentration in the oxygen-limited culture.

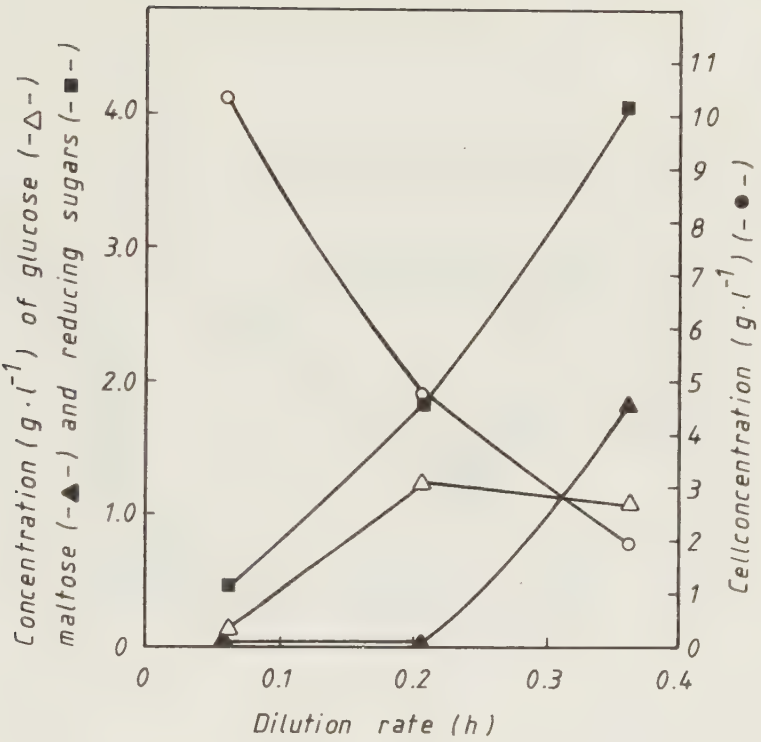

Figure 2 Accumulation of glucose, maltose and reducing sugars in a continuous oxygen-limited culture of S. fibuliger Y76 (aeration 0.05 VVM) as a function of dilution rate.

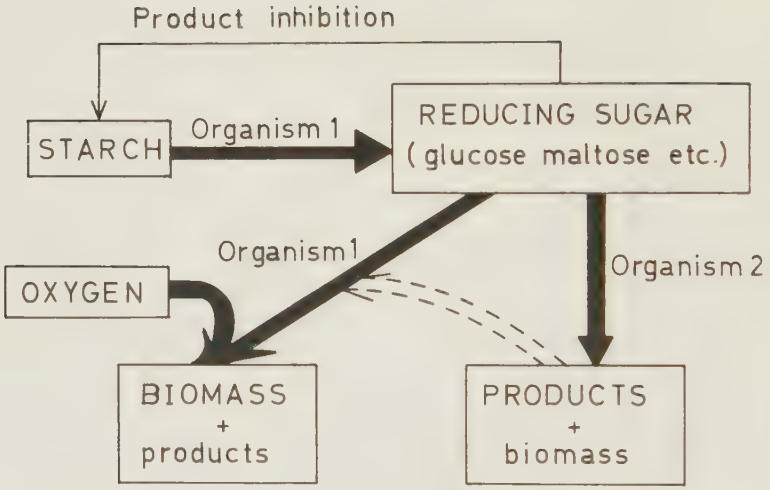

Figure 3 Diagram of the mixed culture system.

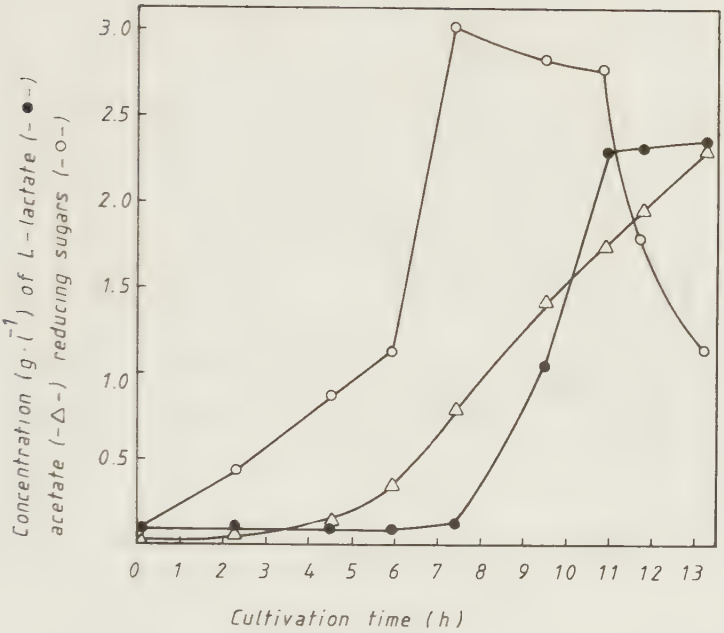

Figure 4 Mixed batch culture of S. lactis 65.1 and S. fibuliger Y76 at a constant, low oxygen supply rate (aeration 0.04 VVM).

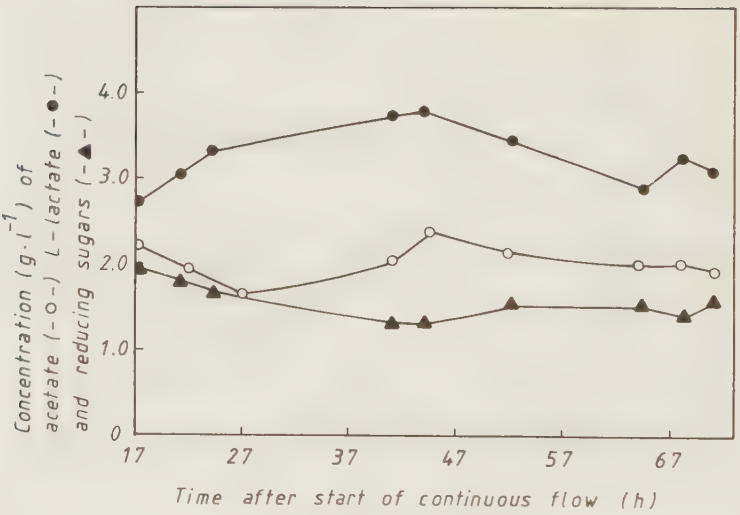

Figure 5 Oxygen-limited mixed continuous culture of S. fibuliger Y76 and S. lactis 65.1 (aeration 0.10 VVM). Dilution rate 0.15 h^{-1}.

VI

Steady-state and transient growth of Streptococcus mutans in continuous culture.

J.C. Leung, M.H. Häggström, C.L. Cooney and A.J. Sinskey

ABSTRACT

We examined the growth of Streptococcus mutans PR-89 in batch culture, glucose-limited steady-state continuous culture and transient continuous culture induced by carbohydrate pulses. Lactic acid was the major product of S.mutans PR-89 when this strain was grown in a defined medium in batch culture. In a glucose-limited steady-state continuous culture, the major end products were lactic acid, ethanol and acetic acid. The transient response of S.mutans PR-89 following a pulse addition of glucose to the culture was characterized by a rapid increase in the specific rate of lactic acid production. An average of 90 % of the pulsed glucose was converted to lactic acid during the transient compared to 20-60 % at steady-state. Inorganic phosphate in vitro inhibits activation of LDH by fructose 1,6-diphosphate. Addition of inorganic phosphate or chloramphenicol to the culture with the pulsed glucose, decreased the increment of lactic acid production during the transient. The effects of inorganic phosphate and chloramphenicol on lactic acid production were additive. The intracellular lactate dehydrogenase level increased in the transient period. The results suggest that the increase in the specific productivity of lactic acid in S.mutans PR-89 was caused by both de novo synthesis and activation of existing LDH.

INTRODUCTION

In recent years Streptococcus mutans has been shown to be

a primary etiological agent of dental caries formation in humans and animals (6, 10, 15, 17). The effectiveness of S.mutans results partly from its acidogenic potential; when grown in glucose or sucrose, it produces large amounts of lactic acid (11). When producing acetic acid and ethanol S.mutans reportedly forms formic acid (16).

Several researchers (2, 5, 6, 9, 12, 16), have used a chemostat to study the growth behavior of S.mutans under steady-state growth conditions; however, little information is available, concerning the growth behavior of S.mutans during transients following sudden environmental changes. Transients of this kind are likely to occur in e.g. the oral cavity. In preliminary studies Cooney et al. (3) observed a rapid extensive conversion of glucose to lactic acid during such transients. Studies of transient responses in microbial cultures provide information on the dynamic behavior of continuous cultures and on the mechanisms involved in the regulation on the metabolic level.

Glucose metabolism in S.mutans proceeds mainly through the Emden-Mayerhof-Parnas pathway and is regulated primarily by lactate dehydrogenase which is activated by fructose 1,6-diphosphate (FDP) (1, 14). The FDP activated L-lactate dehydrogenase in streptococci are reported to be inhibited by orthophosphate (4, 7, 16). The effect of inorganic phosphate on the rate of lactic acid production in vivo has not been reported.

This paper concerns batch and steady-state continuous growth of S.mutans PR-89 and reports in greater detail the response of the organism during transients induced by adding carbohydrate pulses to glucose-limited cultures. The mechanisms involved in regulating the lactate dehydrogenase activity are also discussed.

MATERIALS AND METHODS

Organism and growth conditions. S.mutans PR-89 (Brathall serotype c) was obtained from Dr. Robert Fitzgerald (V.A. Hospital, Miami, Fla). The organism was maintained through stabs in agar containing 30 g/liter trypticase-soy and 5 g/liter yeast extract and stored at 4° C. Temperature and pH were controlled at 37° C and 7.0, respectively; 2.0 N NaOH was used to keep a constant pH.

Growth medium. Cultures for chemostat inoculation were grown in a medium containing (g/liter): trypticase-soy, 30; yeast extract, 5; systeine-HCl, 0.5. Continuous culture experiments were done in a chemically defined medium containing (g/liter): glucose, 2.8; $(NH_4)_2SO_4$, 1.32; glutamic acid, 0.5; KH_2PO_4, 2.0; and (mg/liter): pyridoxine-HCl, 36; nicotinic acid, 6.9; biotin, 0.018; thiamine-HCl, 0.15; riboflavin, 0.6; calcium pantothenate, 3.6; p-aminobenzoic acid, 0.03; cysteine-HCl, 100; NaCl, 10; $FeSO_4 \cdot 7H_2O$, 27.5; $MgSO_4 \cdot 7H_2O$, 250; $MnSO_4 \cdot H_2O$, 16.9; $ZnSO_4 \cdot H_2O$, 2.91; $CuSO_4 \cdot 5H_2O$, 2.5; $CoCl_2 \cdot 2H_2O$, 2.37; $Na_2MoO_4 \cdot 2H_2O$, 2.41; $CaCl_2 \cdot 2H_2O$, 14.7. Glucose was autoclaved separately, and vitamins were sterilized by Millipore filtra-

tion.

Equipment. Continuous culture studies were done in a one-liter chemostat described by White et al. (13). In the fermentor, anaerobiosis was maintained by sparging 50 ml/min of N_2:CO_2 (95:5) gas mixture through the broth.

Determination of growth. Culture turbidity was measured with a Klett-Summerson colorimeter using a red filter (660 nm). Dry cell weight correlated with Klett units by a conversion factor of 0.3 mg dry cell mass/ml for 100 Klett units.

Chemical assays. Glucose concentration was measured with a hexokinase/glucose-6-phosphate dehydrogenase kit (Calbiochem Co., LaJolla, Calif.).

A L-lactate dehydrogenase (L-LDH) based kit (Sigma Chemical Co., St. Louis, Mo) was used to measure lactic acid. D-lactate was assayed in the same way but with the use of a D-LDH (Boehringer, Mannheim, Germany).

Acetic acid and ethanol were measured by gas chromatography on a 1/8 inch I.D., 5-ft-long stainless steel Chromosorb 101 column (Supelco, Inc., Bellefonte,PA) using a Varian 940 gas chromatograph (Varian Aerograph, Waltham, Mass). Nitrogen, the gas carrier was at a flow rate of 40 ml/min. A hydrogen flame ionization detector was used. Ethanol determinations were done at 110° C and acetic acid determinations at 140° C. Methanol and propionic acid were added to the cell-free fermentation

sample as internal standards. The sample pH was 2.0.

Cell-free extract. Cell-free extracts were prepared as follows: thirty milliliters of cell suspension was removed from the continuous culture and immediately mixed with chloramphenicol to give a final concentration of 10 μg/ml. The cells were harvested by centrifugation at 15,000 x g, 4^{o} C for 10 min, washed in 0.04 M sodium phosphate buffer (pH 6.8). The pellet was suspended in 3 ml of the same buffer, 2.5 ml glass beads (0.2 mm) were added, and the cells were disintegrated for 3 times 1 minute on ice in a MSE ultrasonic disintegrator (MSE, Crawley, England). Cell debris was removed by centrifugation at 30,000 x g, 4^{o} C for 30 min. The supernatant was used as cell-free extract. The protein concentration in the cell-free extract was measured by the method of Lowry et al. (8), using bovine serum albumin fraction V (Boehringer, Mannheim, Germany) as standard.

Assay of LDH activity. LDH activity was measured in cell-free extracts. The standard assay system contained 67 mM Tris (hydroxymethyl) aminomethane-hydrochloride buffer (pH 7.0); 0.16 mM NADH; 0.33 mM fructose 1,6-diphosphate tetracyclohexylammonium salt FDP; 33 mM sodium-pyruvate and cell-free extract. The reaction was initiated by adding cell-free extract and was run at 25^{o} C. One unit of LDH activity is defined as the amount of enzyme that oxidizes 1 μmole NADH/min. When pyruvate was omitted from the assay mixture no NADH oxidase activities were detected. Results from assays on duplicate cell-free extracts were in agreement; LDH activity

measurement was proportional to enzyme concentration and an increase of the FDP concentration in the assay system did not increase the LDH activity.

Glucose transient experiments. The glucose pulse experiments were performed after the continuous culture had reached steady-state, i.e., when culture turbidity had remained constant over a period of three cell doubling times or after an interval, equivalent to three times the residence time, had passed since the last disturbance. Five milliliters of concentrated glucose solution was injected into the fermentor. During the transient following the pulse addition, 4-ml samples were taken every 15-30 min for turbidity measurement and substrate and product analyses. In the experiment using inorganic phosphate, 5-ml concentrated sodium phosphate solution was added, to give different final phosphate concentrations ranging from 26 to 100 mM. Chloramphenicol, mixed with the glucose solution used for the pulse, was added. The final concentration of chloramphenicol in the fermentor immediately after the pulse was 25 mg/liter.

Data analysis. Specific productivity of product formation (q_p) for batch culture is calculated using the following relationship:

$$q_p = \frac{1}{x} \frac{dP}{dt} \quad \text{(mg product formed/mg cell·h)}$$

where P is product concentration (mg/ml), and x is the con-

concentration of cell mass (mg dry weight/ml). For continuous culture, the specific productivity of product formation is calculated using the relationship:

$$\frac{dP}{dt} = q_p x - DP$$

and therefore,

$$q_p = \frac{1}{x}\frac{dP}{dt} + \frac{DP}{x}$$

where D denotes the dilution rate (h^{-1}).

Total carbon recovery is calculated assuming the cells contain 50 % carbon by dry weight and that the molar concentration of formic acid is the sum of the molar concentrations of ethanol and acetic acid.

RESULTS

Batch culture. S.mutans was grown in batch culture using a chemically defined medium (Fig. 1). The initial glucose concentration was 2.9 g/liter. Lactic acid production approximatively parallelled growth; at the point of maximum lactic acid concentration, total conversion of glucose to lactic acid was 77 % by weight. A carbon balance using cell mass 0.3 mg dry weight/100 Klett units) and lactic acid as the only fermentation products showed a 94 % recovery. The specific growth rate, and specific lactate production rate

during batch growth were calculated from the data in Fig. 1. The specific growth rate rised to a maximum value of 0.58 h^{-1}. However, the specific productivity of lactic acid remained at about 2.7 mg lactic acid/mg cell/h throughout the growth period and was independent of specific growth rate.

Steady-state continuous culture. The steady-state growth of S.mutans was examined at various dilution rates, ranging from 0.03 h^{-1} to 0.19 h^{-1} (Fig. 2). The feed glucose concentration was 3.0 mg/ml and the residual glucose concentration was always below 0.05 mg/ml. L-lactic acid was the major fermentation product. However, significant amounts of ethanol and acetic acid were also produced; their concentrations remained relatively constant within the range of dilution rate studied. The conversion yield of glucose to cell mass and products was calculated on a weight basis (Fig. 3). The percentage of carbon recovery increased from 80 % at dilution rate 0.03 h^{-1} to 98 % above 0.15 h^{-1}. The low carbon recovery obtained at low dilution rate was probably caused by formation of unidentified products. The q_p for ethanol and acetic acid were constant at all dilution rates. However, the q_p of lactic acid increased from 0.1 to 0.8 mg/mg-h when the dilution rate was increased from 0.03 to 0.19 h^{-1} compared with the specific productivity of lactic acid (2.7 mg/mg-h) observed in the batch culture at a specific growth rate of 0.58 h^{-1}.

Transient response of a steady-state culture. A pulse of glucose was added to a continuous steady-state culture at different dilution rates and the transient response was

studied. Table 1 summarizes steady-state parameters prior to the pulse and results after the pulse. The culture response to a glucose pulse at different dilution rates were qualitatively very similar (Fig. 4). Initially, the glucose was utilized rapidly and lactic acid concentration increased rapidly as glucose was depleted. Ethanol concentrations and acetic acid increased slightly. Figure 5 shows the specific productivity of lactic acid and the conversion yield of lactic acid from glucose. The results are typical of all runs. Specific productivity increased rapidly several-fold after glucose addition; the conversion yield of glucose to lactic acid increased from a steady-state value of approximately 50 % to almost 100 %, the theoretical yield. Overall conversion of glucose to lactic acid during the transient was over 90 % for all these experiments (Table 1), compared with conversions of 20-60 % during steady-state and 77 % in batch culture. The conversion yields of ethanol or acetic acid during the transients did not vary significantly from their corresponding steady-state values. The specific productivity of lactic acid increased markedly (0.29 to 3.40 mg/mg·h) during the transient period and reached values over the maximum specific productivity observed in batch culture.

Regulation of lactic acid production. The fructose 1,6-diphosphate activated L-lactate dehydrogenases are inhibited by orthophosphate *in vitro* (4, 7, 16). The *in vivo* transient response following a pulse addition of glucose together with various amounts of orthophosphates was studied (Fig. 6). The increase in the specific productivity of lactic

acid observed after a glucose pulse was reduced by the presence of inorganic phosphate; the magnitude of the reduction decreased with increasing amounts of phosphate added.

When the protein synthesis inhibitor chloramphenicol was added together with glucose the specific productivity of lactic acid increased rapidly but the maximum value was only about 50 % of that obtained when glucose was added alone (Fig. 7). The addition of various amounts of orthophosphate together with glucose and chloramphenicol further decreased the observed increase in specific lactic acid production (Fig. 8).

These transient responses showed that inorganic phosphate and chloramphenicol had an additive effect on the specific lactic acid production during the transient (Table 2). When chloramphenicol was added alone, a 50 % reduction in maximum specific productivity was observed (e.g., from 2.0 to 1.0 mg/mg-h), compared to the transient response induced by a glucose pulse alone. Similarly, 18 and 25 % reductions in the maximum specific productivity of lactic acid were measured when inorganic phosphate was added at concentrations of 50 and 100 mM, respectively. The additive effect of chloramphenicol and inorganic phosphate could be seen when these inhibitors were added together with the glucose pulse. A 63 % reduction in maximum specific productivity of lactic acid (from 2.0 to 0.75 mg/mg-h) was the result when 50 mM phosphate and 25 mg/liter of chloramphenicol were added with the glucose pulse. Similarly, a 75 % reduction (from 2.0 to 0.53 mg/mg-h) was

observed when 100 mM phosphate and 25 mg/liter of chloramphenicol were added. These reductions were equal to the sum of the individual decreases that occur when the inhibitors were added alone. However, during transients induced by adding inhibitors, the increase in conversion yield of lactic acid from glucose was unaffected. The overall lactic acid yield from glucose increased to over 95 % from a pre-addition steady-state value of roughly 30 %. Little or no increase was detected in ethanol or acetic acid during these transients.

As phosphate is known to inhibit activated lactate dehydrogenases *in vitro* the observed effect of phosphate on the *in vivo* activity of the cells strongly suggest that the activity of the lactate dehydrogenase increases due to an increased FDP level and that phosphate is inhibiting the activation of the LDH in the cell. The reduced increase in lactic acid production in the presence of chloramphenicol suggested that *de novo* synthesis may be involved in the regulation of the LDH and thus in the lactic acid production rate. An experiment was therefore carried out where the LDH activity of the cells was measured during the transient period following a pulse addition of glucose to a steady-state continuous culture at the dilution rate 0.08 h^{-1}. Intracellular LDH level increased rapidly, corresponding to the increased specific productivity of lactic acid (Fig. 9). The results show that *de novo* synthesis of LDH is involved in the regulation of the lactate dehydrogenase activity of the cells.

DISCUSSION

Yamada and Carlsson (16) have shown that the LDH of S.mutans JC2 is dependent on FDP and that there is a high intracellular FDP concentration under glucose-excess conditions. In glucose-limited conditions, FDP concentration is low. As the cells were starved of glucose during the harvesting procedure used the FDP values reported are most likely to low. Yamada and Carlsson (16) have also shown that LDH levels in S.mutans JC2 are in the same order of magnitude whether the strain is grown under glucose-limited or glucose-excess conditions. They concluded that intracellular FDP is a primary regulator affecting relative formation of fermentation products (16). However, in our transient response study of S.mutans, the results suggest that two control mechanisms affect the enzyme LDH.

During the transient condinuous culture, previously slow-growing cells (i.e., D = 0.03 - 0.06 h^{-1}) reached a specific lactic acid productivity approaching the batch culture value, and previously fast-growing cells (i.e., D = 0.11 - 0.19 h^{-1}) reached values substantially higher than the batch culture value (Table 1). On the other hand, when chloramphenicol was added to suppress protein synthesis during the transient a markedly decreased lactic acid production was observed (Fig. 7). These results suggested that in addition to LDH activation, LDH level may increase during the transient. Measurement of intracellular LDH activities during the transient period verified that de novo synthesis of LDH was involved

in controlling lactic acid production.

Further evidence for simultaneous regulation of LDH activity and synthesis was obtained by studying transient response to a glucose pulse in the presence of inorganic phosphate. High phosphate concentrations have been shown to increase the half-saturation constant, K_m, for FDP, the enzyme activator, in streptococci (4, 7, 16). The responses of S.mutans in vivo to glucose pulses in the presence of inorganic phosphate confirmed these findings. This inhibitory effect was also reflected in the decreasing maximum specific productivity of lactic acid during transients with increasing phosphate concentrations (Table 2). Transient responses induced by adding a glucose pulse with inorganic phosphate and chloramphenicol further reduced the increase in specific productivity of lactic acid. These reductions were more pronounced than those found when chloramphenicol or inorganic phosphate was administered alone (Table 2); they may be explained as the sum of chloramphenicol effects on LDH synthesis and inorganic phosphate effects on FDP activation of existing LDH. The regulatory mechanism of LDH enzyme can also be used to explain the results of the steady-state continuous culture.

In continuous culture, our results show that the specific productivity of lactic acid before the transient was only a fraction of the maximum activity observed in batch culture (e.g., 2.7 mg/mg cell-h). This observation can be explained as follows. When growing in batch culture with glucose as the carbon source, S.mutans PR-89 has a high intracellular FDP

level, which activates LDH to operate at a higher rate. Pathway regulation is affected only when the glucose supply is limited and the low intracellular FDP concentration decreases the existing LDH activity.

These findings may be applied to other fermentation processes in which anaerobic fermentation products are formed, i.e., lactate formation by dairy starter cultures and ethanol formation by yeast. In cases where low yields are routinely observed, nutrient pulsation might be used to enchance the overall yield of a desired product.

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Table 1. A summary of the glucose-pulse transients and a comparison to the steady state values prior to the pulse

Dilution rate (h^{-1})	Glucose added (mg/ml)	Yields of products (mg/mg glu)						Specific productivity of lactic acid (mg/mg-h)	
		Lactic acid		Ethanol		Acetic acid			
		S.S.[a]	Overall[b]	S.S.[a]	Overall[b]	S.S.[a]	Overall[b]	S.S.[a]	Maximum during transient
0.03	1.57	0.30	0.95	0.13	0	0.08	0.03	0.11	2.0
0.08	1.71	0.23	0.85	-	-	-	-	0.15	2.0
0.11	1.93	0.38	0.90	0.10	0.04	0.05	0.01	0.29	3.4
0.19	2.02	0.59	0.90	0.05	0	0.06	0.04	0.78	3.9

[a]Steady state

[b]Over a period of 3 h immediately after the carbohydrate pulse

Table 2. A summary of glucose-pulse transients involving chloramphenicol and inorganic phosphate

($D = 0.08\ h^{-1}$)

Pulse	Glucose added (mg/ml)	Yield of lactic acid (mg/mg glu)		Specific productivity of lactic acid (mg/mg-h)	
		S.S.[a]	T.S. overall[b]	S.S.[a]	Maximum during transient
Glucose	1.71	0.23	0.85	0.11	2.00
Glucose + CAP[c]	1.70	0.30	0.88	0.14	1.00
Glucose + 26 mM P[d]	1.64	0.42	0.88	0.17	1.80
Glucose + 50 mM P	1.69	0.30	0.85	0.12	1.62
Glucose + 100 mM P	1.88	0.22	0.85	0.09	1.48
Glucose + 50 mM P + CAP	2.11	0.22	0.91	0.08	1.75
Glucose + 100 mM P + CAP	1.99	0.29	0.76	0.12	0.53

[a] Steady state

[b] Transient state over a period of 2 h

[c] Chloramphenicol (conc. 25 mg/l)

[d] Phosphate

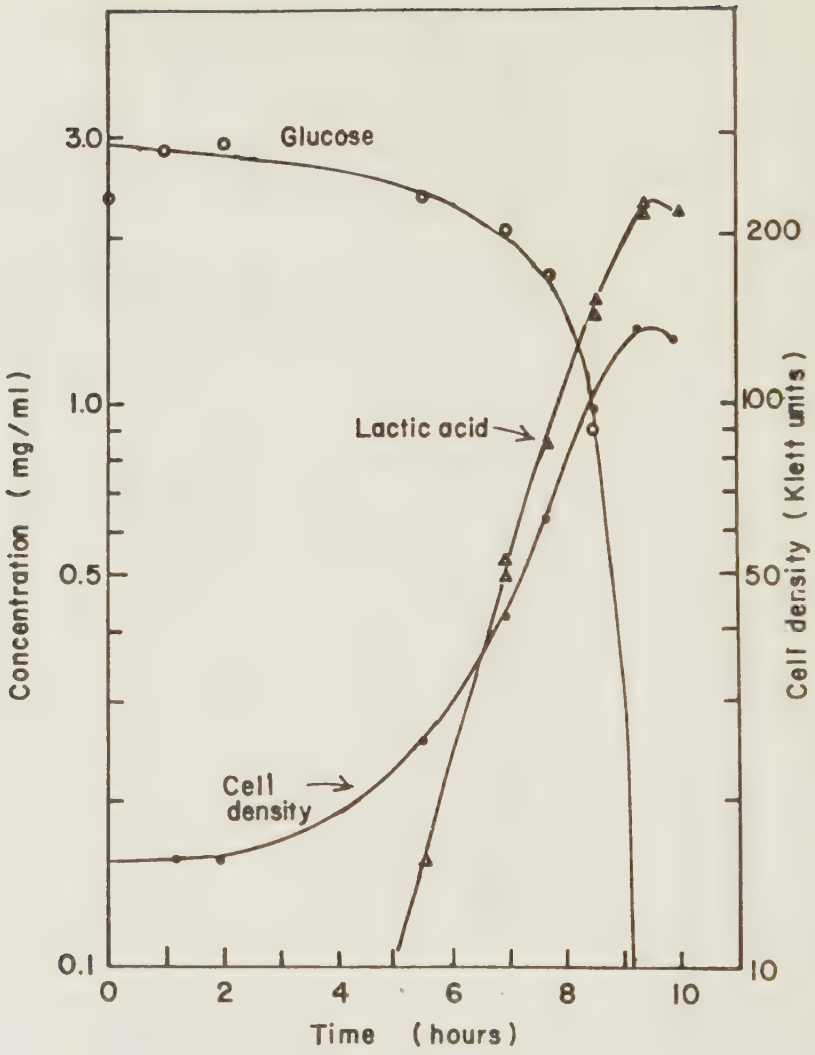

Figure 1 Growth of S.mutans in batch culture on a defined medium (from Ref. 3).

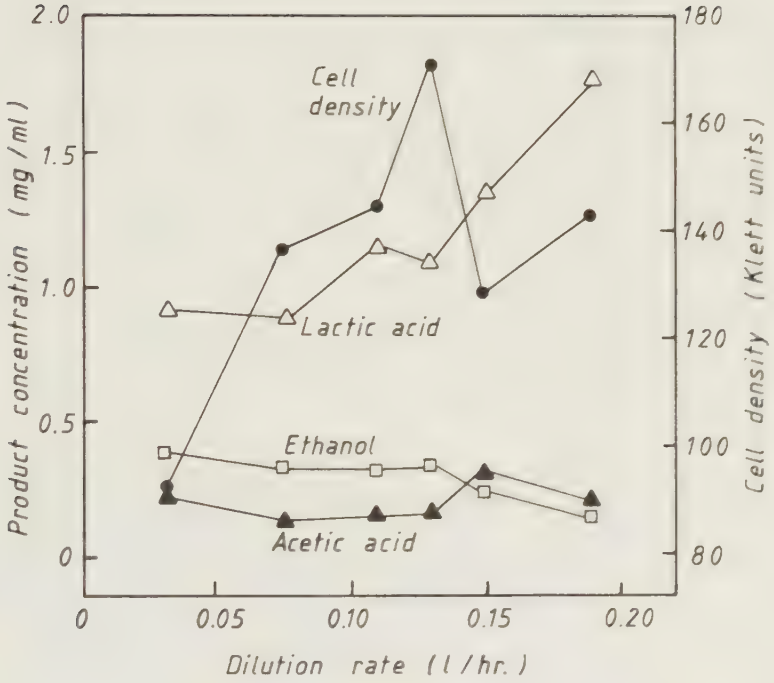

Figure 2 Results from a glucose-limited continuous culture of S. mutans at steady-states.

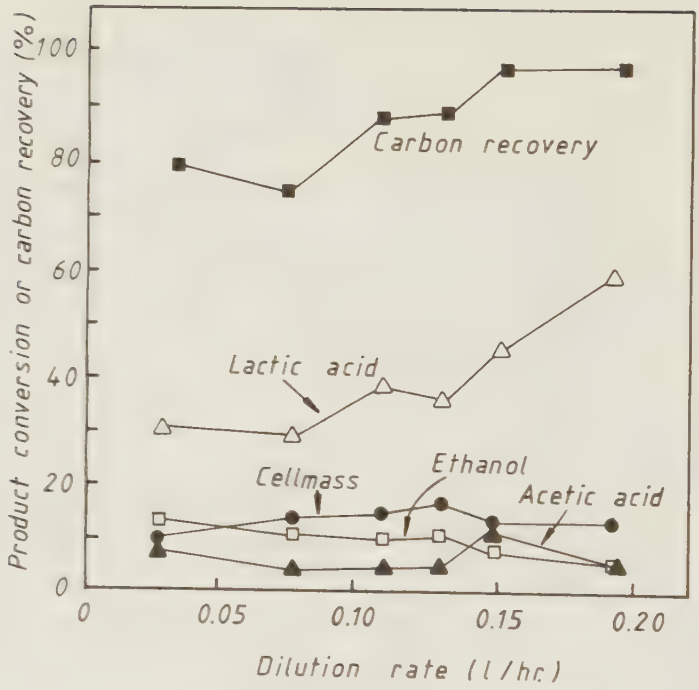

Figure 3 Conversion of utilized glucose to cell mass and products by S.mutans at steady-states; total carbon recovery calculated from the products and cell mass.

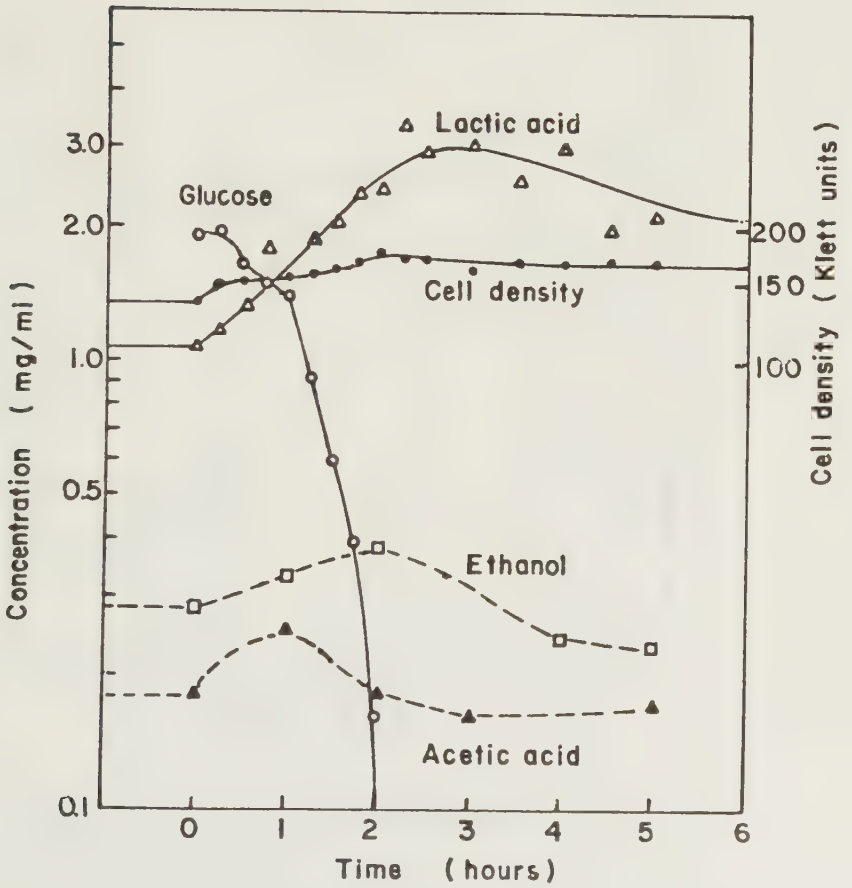

Figure 4 Transient response of S.mutans PR-89 to pulse addition of 1.93 mg/ml of glucose at time = 0. The dilution rate is 0.11 h^{-1} and glucose was the limiting nutrient.

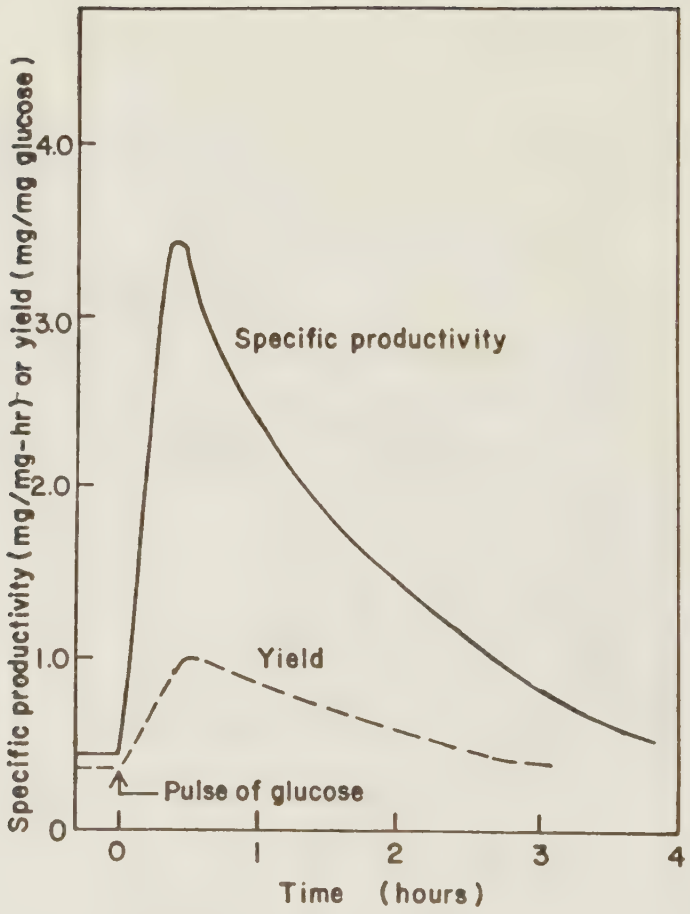

Figure 5 Lactic acid yield from glucose and specific productivity of lactic acid during transient response of a glucose-limited S.mutans culture to the pulse addition of glucose. The dilution rate is 0.11 h^{-1}.

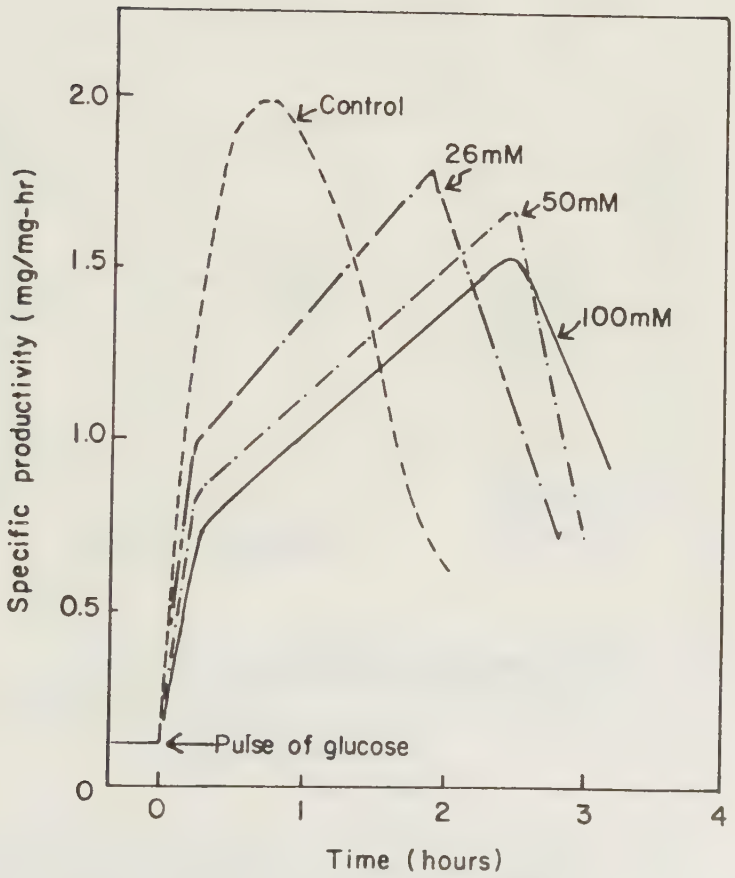

Figure 6 Specific lactic acid productivity during transient responses of a glucose-limited S.mutans culture to pulse additions of glucose plus various concentrations of inorganic phosphate. The dilution rate is 0.08 h^{-1}.

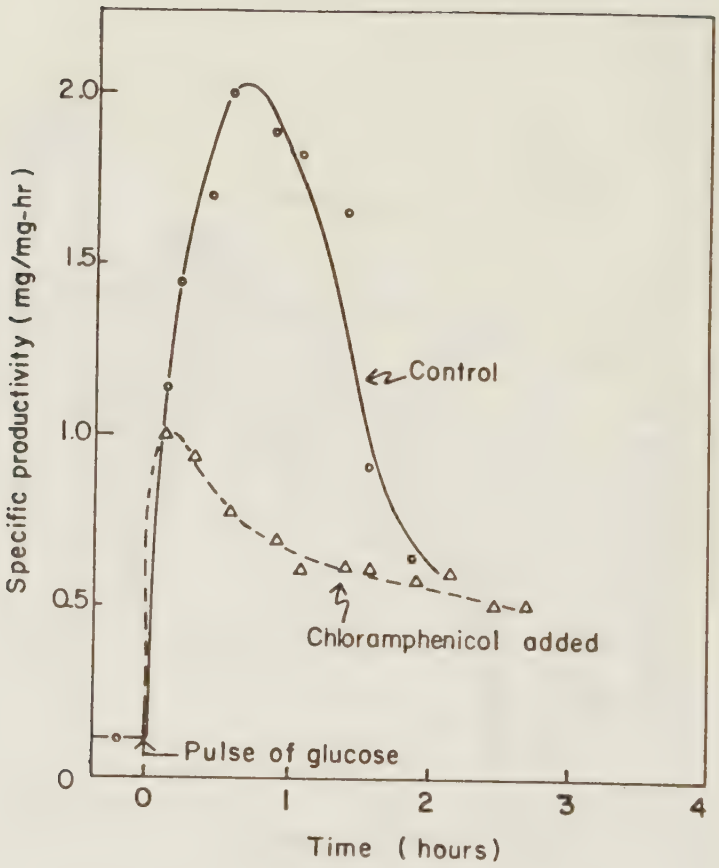

Figure 7 Specific lactic acid productivities during transient responses of a glucose-limited S.mutans culture to pulse additions of glucose and glucose plus chloramphenicol. The dilution rate is 0.08 h^{-1}.

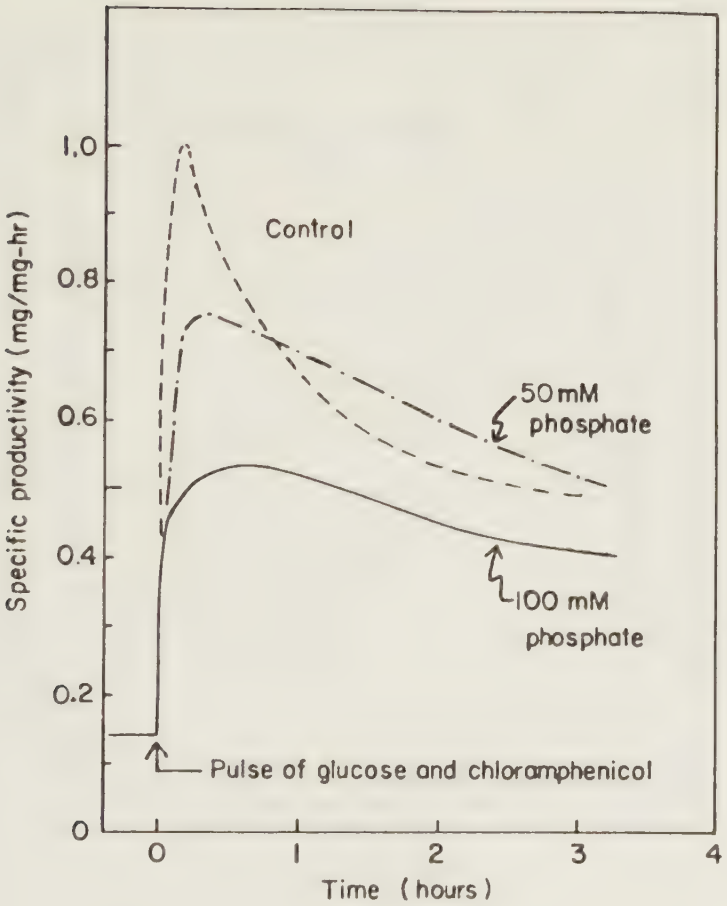

Figure 8 Specific lactic acid productivities during transient responses of a glucose-limited S.mutans culture to pulse additions of glucose with chloramphenicol. Different concentrations of inorganic phosphate were added as noted. The dilution rate is 0.08 h^{-1}.

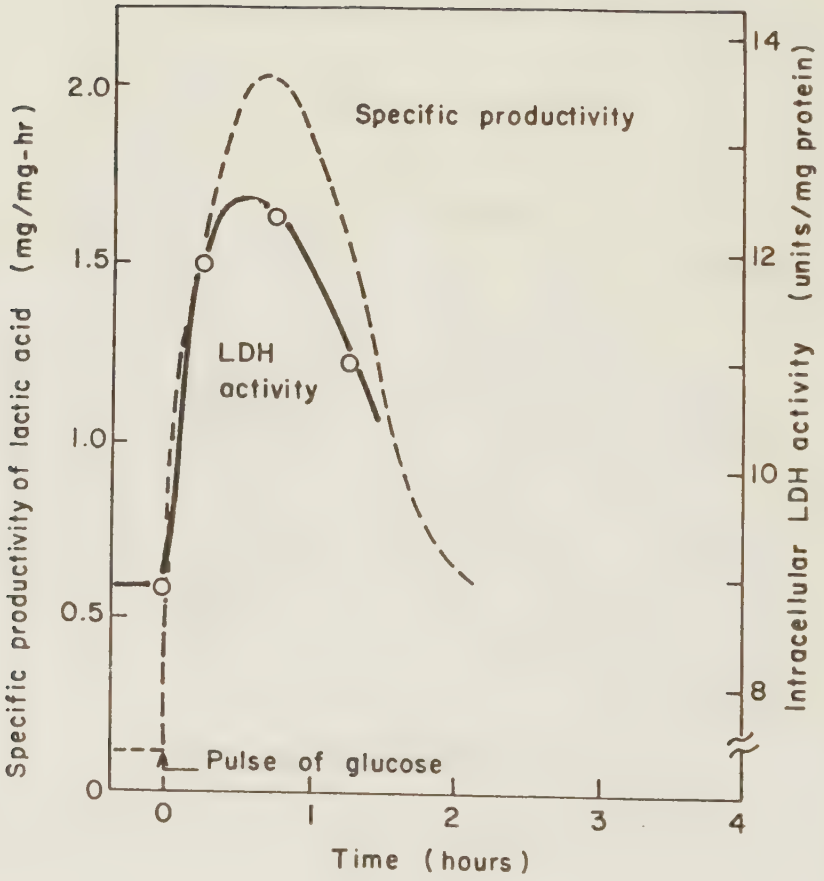

Figure 9 Specific productivities of lactic acid and intracellular LDH activity during transient responses of a glucose-limited S.mutans culture to pulse additions of glucose. The dilution rate is 0.08 h^{-1}.